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STUDIES OF PLATELET FUNCTION, AND EFFECTS OF ASPIRIN AND CLOPIDOGREL TREATMENT

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ABSTRACT

Antiplatelet therapy with aspirin and clopidogrel is a cornerstone in cardiovascular prevention and lowers cardiovascular mortality and morbidity in patient with coronary artery disease (CAD). Mental stress and physical exertion can trigger acute coronary events and prothrombotic responses to stress may contribute to such triggering. Platelet activation seems to contribute to the pathogenesis of preeclampsia.

The dose- and time-dependence of antiplatelets effects of aspirin (37.5 mg/day, 320 mg/day and a single dose of 960 mg) were studied in 15 male healthy volunteers. The variable most sensitive to inhibition by aspirin was arachidonic acid (AA)-induced platelet aggregation in platelet rich plasma, followed by serum TxB₂. The urinary excretion of 11-dehydro-TxB₂ was less markedly, but dose-dependently reduced by aspirin. The effectiveness of aspirin is thus highly dependent on the assay method used. There was limited inhibition by aspirin of collagen and AA-induced platelet aggregation in hirudinized whole blood and there was recovery of platelet function in whole blood within a normal 24 h dosing interval in healthy volunteers.

The effects of clopidogrel treatment on exercise-induced platelet activation were examined in 15 healthy volunteers who performed an exhaustive exercise test, and in 31 aspirin treated patients with stable CAD who performed a symptom limited exercise test in a randomized, double-blind, placebo controlled study. Strenuous exercise evoked multicellular activation in vivo and promoted a prothrombotic state. Clopidogrel treatment inhibited platelet and platelet-leukocyte aggregation evoked by ADP and thrombin (which releases ADP from platelets) stimulation in vitro but the acute prothrombotic response to exercise was little influenced by clopidogrel treatment in both aspirin treated CAD patients and healthy volunteers. This indicates that the P2Y₁₂ receptor is not likely to be of major importance for “stress-induced” platelet activation.

The urinary excretion of 11-dehydro-TxB₂ (TxM) reflects platelet activity in vivo. The present work improved the methodology for immunological measurements of TxM in urine, and also showed that such measurements may be performed in plasma for the evaluation of platelet activity. This method showed excellent agreement with the gold standard method based on gas chromatography-mass spectrometry.

Urinary TxM increased throughout normal pregnancy with the highest excretion 3-7 days postpartum. In 28 patients at high risk of suffering preeclampsia urinary TxM was elevated compared to the healthy pregnant women already before gestational week 13. Low-dose aspirin treatment (75 mg once daily) effectively inhibited platelet-dependent thromboxane production early in pregnancy in high risk patients, but urinary TxM increased later during pregnancy. The results support a role for thromboxane in preeclampsia, and suggest that aspirin treatment should be initiated earlier than has been the case in studies which show only modest protective effects of low-dose aspirin.

This thesis shows that both aspirin and clopidogrel have limitations as antithrombotic agents. Some patients at risk may not benefit from adequate aspirin protection against cardiovascular events. One explanation for this result may be our finding of a recovery of platelet function 24 hours after dosing and that pronounced AA-induced platelet aggregation persists in whole blood despite aspirin treatment. “Stress-induced” platelet activation is little influenced by treatment with aspirin and clopidogrel. This information about treatment with aspirin and clopidogrel might be useful in shaping more efficient antithrombotic therapy for patients at high cardiovascular risk.

LIST OF PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-V.

- I. Perneby C, Granström E, Beck O, Fitzgerald D, Harhen B, Hjemdahl P. Optimization of an enzyme immunoassay for 11-dehydro-thromboxane B₂ in urine: comparison with GC-MS. *Thromb Res* 1999;96:427-436.
- II. Perneby C, Wallén NH, Rooney C, Fitzgerald D, Hjemdahl P. Dose- and time-dependent antiplatelet effects of aspirin. *Thromb Haemost* 2006;95:652-658.
- III. Perneby C, Vahter M, Åkesson A, Bremme K, Hjemdahl P. Thromboxane metabolite excretion during pregnancy – influence of preeclampsia and aspirin treatment. *Thromb Res* 2011;127:605-606.
- IV. Perneby C, Wallén NH, Hu H, Li N, Hjemdahl P. Prothrombotic responses to exercise are little influenced by clopidogrel treatment. *Thromb Res* 2004;114:235-243.
- V. Perneby C, Wallén NH, Hofman-Bang C, Tornvall P, Ivert T, Li N, Hjemdahl P. Effect of clopidogrel treatment on stress-induced platelet activation and myocardial ischemia in aspirin-treated patients with stable coronary artery disease. *Thromb Haemost* 2007;98:1316-1322.

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LIST OF ABBREVIATIONS

Aspirin	Acetylsalicylic acid
ADP	Adenosine-diphosphate
AA	Arachidonic acid
β -TG	β -thromboglobulin
CABG	Coronary artery by pass grafting
CAD	Coronary artery disease
COX	Cyclooxygenase
CVD	Cardiovascular disease
EIA	Enzyme immunoassay
ECG	Electrocardiographic recordings
GC-MS	Gas chromatography-mass spectrometry
GP	Glycoprotein
LTA	Light transmittance aggregometry
MFI	Mean fluorescence intensity
MI	Myocardial infarction
NO	Nitric oxide
PCI	Percutaneous coronary intervention
PGG ₂	Prostaglandin G ₂
PGH ₂	Prostaglandin H ₂
PGH	Prostaglandin H
PGI-M	2, 3-dinor-6-keto-prostaglandin F _{1α}
PGI ₂	Prostacyclin
PLA	Platelet-leukocyte aggregates
PPAs	Platelet-platelet aggregates
PPI	Proton pump inhibitors
PRP	Platelet rich plasma
TF	Tissue factor
TNF- α	Tumour necrosis factor- α
TP	Thromboxane-Prostaglandin H ₂ -receptor
TxB ₂	Thromboxane B ₂
TxA ₂	Thromboxane A ₂
vWF	von Willebrand factor

1 INTRODUCTION

1.1 GENERAL BACKGROUND

Cardiovascular disease (CVD) is the leading cause of death in the United States and Western Europe [1]. The discovery of the importance of platelets in CVD has changed therapy. Antiplatelet agents, especially acetylsalicylic acid (aspirin), are today cornerstones in the prevention of atherothrombotic complications.

Atherosclerosis is a chronic inflammatory disease [2] and intravascular thrombosis occurs when an atherosclerotic plaque rupture or fissure leads to exposure of platelet activating and procoagulant elements [3-6]. Platelets interact with various endothelial and inflammatory mechanisms [6] and a critical component of atherothrombosis may be the activation of platelets by inflammatory triggers. Mental stress and physical exertion activate platelets and prothrombotic responses to “stress” may contribute to the triggering of acute coronary events [7-13]. In addition, frequent episodes of stress with haemostatic activation may contribute to the slow progression of atherosclerosis [13,14].

Platelets are essential for primary hemostasis and repair of the endothelium [4]. The ability of platelets to participate in both normal hemostasis and atherothrombosis depends on their adhesive properties and their capacity to become activated and release various compounds with biological activities in response to various stimuli [15].

Aspirin (i.e., low-dose acetylsalicylic acid) and clopidogrel are widely used and very well documented antiplatelet drugs that lower cardiovascular morbidity and mortality [16-18]. However, the occurrence of adverse ischemic events despite antiplatelet treatment is a serious clinical problem which, in part, may be related to a poor treatment response. There is variability in responses to treatment with aspirin and clopidogrel and the possibilities of tailoring antiplatelet therapy based on platelet function testing is a very active research field at present. However, the evaluation of antiplatelet treatment with different platelet function tests has not yet been sufficiently documented for clinical use [19-21]. The relationship between surrogate markers for platelet function and clinical events is a critical issue. Thrombosis involves multiple signalling pathways and treatment failure is not synonymous with drug resistance [19,20]. There are thus no agreed definitions of aspirin or clopidogrel “resistance” and the mechanisms of resistance as well as how to tailor the treatment remains incompletely defined [21,22].

Preeclampsia is a multisystem disorder in which platelet activation seems to contribute to the pathogenesis [23,24]. Several studies have therefore evaluated the effects of aspirin treatment in the primary prevention of preeclampsia, but the results have been relatively disappointing [25].

The present work concerns platelet function and the effects of antiplatelet treatment in healthy volunteers, in patients with CVD, and in pregnant women at high risk of suffering preeclampsia.

1.2 PLATELET PHYSIOLOGY

Platelets are small discoid cell fragments which circulate in large numbers ($200-300 \times 10^9/L$ - under normal conditions). Platelets are shed from megakaryocytes in the bone marrow. Their lifespan is normally 9-11 days and they have a median volume of 7-9 fL [26]. The main physiological function of platelets is to arrest bleeding from damaged blood vessels through the formation of hemostatic plugs. Platelets are sequestered in the spleen which retains approximately one-third of the body's platelets in a platelet pool [27]. There are three different platelet specific secretory granules: α -granules, dense granules and lysosomes. Examples of proteins stored in α -granules are P-selectin, von Willebrand factor (vWF), β -thromboglobulin (β -TG), fibrinogen, GPIIb/IIIa, Factor V, Factor X, and CD40 ligand. The dense granules contain adenosine-diphosphate (ADP), serotonin and Ca^{2+} which promote platelet activation. Lysosomes contain hydrolytic enzymes.

The platelet cytoplasmic membrane is composed of a bilayer of polarized phospholipids containing arachidonic acid (AA) which is released and converted to thromboxane A_2 (TxA_2) during platelet activation. The external layer of the platelet membrane contains numerous glycoproteins (e.g., GPIIb/IIIa, GPIb/IX/V, and GPVI) which act as receptors for various ligands and are essential for adhesion and aggregation. GPIIb/IIIa is the most abundant receptor and belongs to the integrin family of heterodimeric adhesion molecules [28]. A normal platelet contains approximately 80 000 GPIIb/IIIa complexes [29]. Upon activation GPIIb/IIIa undergoes a conformational change enabling the binding of its principal ligand fibrinogen which is crucial for platelet aggregation. The cytoplasmic membrane invaginates into the platelet to form the open canalicular system. This system is a storage site for membrane receptors, enlarges the platelet surface area and facilitates the release of granular contents. The platelet also has a dense tubular system which is a site for Ca^{2+} storage and various enzymatic activities including those required for prostaglandin synthesis.

1.3 PLATELET ACTIVATION

At a site of vascular injury, platelets are exposed to thrombogenic components of the subendothelium (collagen, vWf, fibronectin and tissue factor; TF). This is the first step of primary hemostasis. The endothelium normally has antithrombotic properties, but inflammation, infection and metabolic alterations decrease the antithrombotic potential of endothelial cells normally mediated by compounds like prostacyclin (PGI_2) and nitric oxide NO [15].

One of the most important ligands for adhesion is the multimeric subendothelial vWF which binds to a platelet receptor; membrane bound glycoprotein (GPIb-IX-V) [30]. This adhesion leads to shape change of the platelet from a smooth disc to a tiny sphere with pseudopodia, and spreading of the platelet on the damaged vessel wall. Signals from adhesion receptors act in synergy with signals from agonists like TxA_2 , adrenaline and ADP through a network of signalling pathways. TxA_2 and ADP provide important positive feedback to reinforce platelet aggregation. ADP elicits its effects on the platelet through $P2Y_1$ and $P2Y_{12}$ receptors [31]. ADP-induced shape change is mediated by $P2Y_1$ receptors coupled to a G-protein and PLC –mediated intracellular calcium elevation [31,32]. The $P2Y_{12}$ receptor is crucial for several platelet functions such as fibrinogen receptor activation [33], dense granule secretion [34], and ADP-mediated generation of TxA_2 [35]. Activation of the $P2Y_{12}$ receptor causes inhibition of adenylylcyclase and potentiates platelet activity by suppressing cyclic AMP formation

[36]. There are about 500-1000 binding sites for ADP on the platelet surface, one third are P2Y₁ and two thirds P2Y₁₂. This is about half of the number of receptors for potent agonists such as thrombin and TxA₂ [37,38].

TxA₂ acts on the specific G-protein coupled Thromboxane-Prostaglandin H₂ (TP) receptor on the platelet surface causing PLC-signalling and platelet activation [31]. As noted above there are approximately twice as many TxA₂ receptors as there are ADP receptors on the platelet. Following shape change the platelet GPIIb/IIIa receptors undergo activation elicited by a variety of agonists such as TxA₂, ADP and thrombin. The receptors for these agonists act through G-proteins and “inside-out” signaling which initiates interactions with the cytoplasmic tail of the GPIIb/IIIa receptor. This causes a conformational change in the extracellular domain of the receptor from a low-affinity to a high-affinity state [39] and allows binding of its principal ligand fibrinogen. This process is strongly dependent of elevation of cytosolic Ca²⁺ [39]. Binding of fibrinogen to the GPIIb/IIIa receptor leads to clustering and generation of “outside-in” in signalling which initiates several intraplatelet responses and promotes the assembly of a procoagulant surface for binding and steric arrangement of coagulation factors resulting in enhanced thrombin generation [40].

Atherosclerotic plaque rupture exposes tissue factor (TF) to the circulation which activates the coagulation system leading to local thrombin generation. Thrombin is the most potent endogenous platelet activator and binds to specific thrombin receptors (PAR-1 and PAR 4) which activates platelets and also results in positive feed-back via release of platelet activating factors like ADP and TxA₂ [41-43]. Thrombin activates platelets at lower concentrations than those required to convert fibrinogen to fibrin and is very important in platelet recruitment during various thrombotic conditions [43,44].

Activated platelets shed microparticles from their membrane surfaces. Platelet-derived microparticles are highly procoagulant and catalyze the formation of thrombin around platelet microaggregates. Thrombin then converts fibrinogen to fibrin. The deposition of fibrin around platelet aggregates leads to consolidation of the thrombus [45]. Stability of the platelet aggregates and their rate of growth are important in determining whether a thrombus will be occlusive or will be dissolved by the blood flow and anti-aggregatory compounds released from the vessel wall [15].

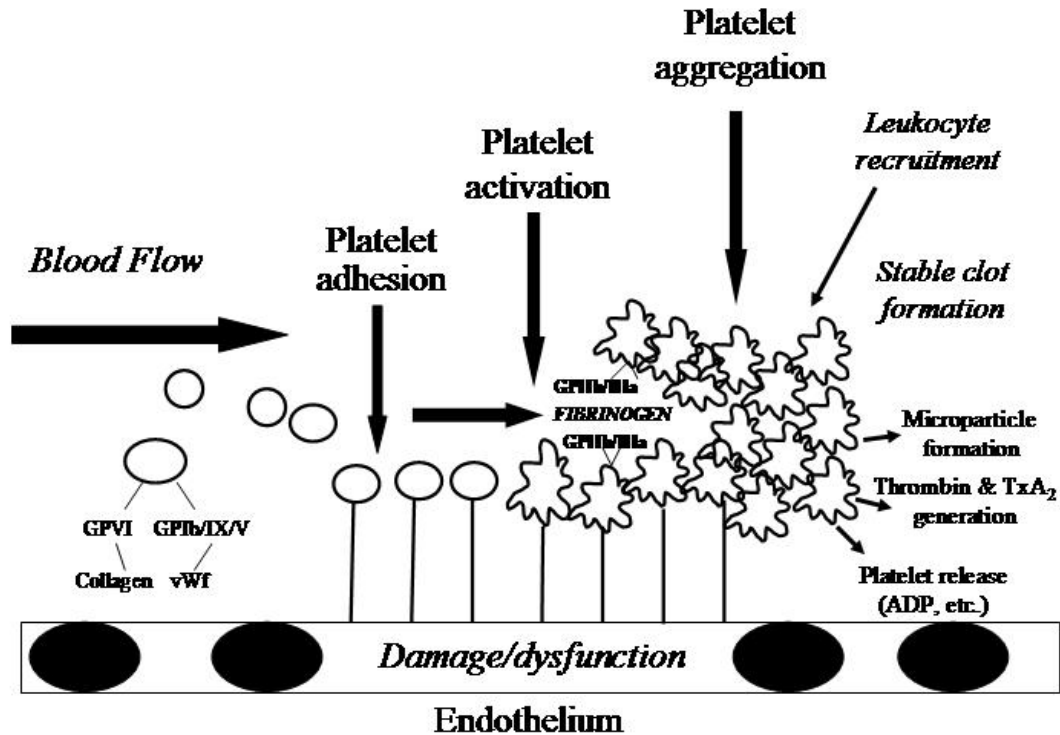


Figure 1: Schematic representation of platelet activation in an atherosclerotic vessel.

1.3.1 Thromboxane biosynthesis

Upon platelet activation arachidonic acid (AA) is cleaved from the platelet membrane by phospholipase A₂. AA is then oxygenated by Prostaglandin H synthase (PGH) into the endoperoxides prostaglandin G₂ (PGG₂) and PGH₂. PGH-synthase has both cyclooxygenase (COX) and hydroperoxidase activity and is commonly termed COX [46]. COX exists in two isoforms of which COX-1 dominates in platelets [46]. PGH₂ is further converted to TxA₂ by a separate enzyme. TxA₂ is the predominant product formed from AA in human platelets [47] and is a very potent vasoconstrictor and platelet agonist. However, TxA₂ is also very unstable and is rapidly (within approximately 30 seconds) converted to TxB₂, and then further metabolized to the stable metabolites 11-dehydro-TxB₂ and 2,3-dinor-TxB₂.

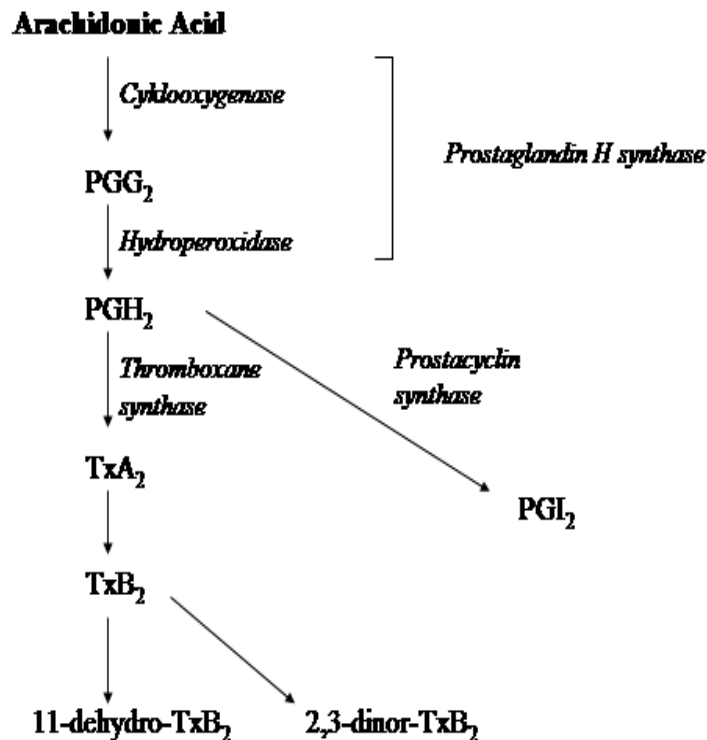


Figure 2: Biosynthesis of thromboxane and metabolites (modified from [48,49]).

TxA₂ is released episodically and has local effects. Its stable metabolites are excreted in urine 15-60 minutes after TxA₂ biosynthesis in vivo. The capacity of human platelets to synthesize and release TxA₂ when maximally stimulated ex vivo may be as much as 5000 times higher than the basal TxA₂ production [50,51]. Episodic increases in 2,3-dinor-TxB₂ and 11-dehydro-TxB₂ excretion have been detected in unstable angina [51,52,53] and during percutaneous coronary intervention (PCI) [53].

Measurements of stable metabolites of TxB₂ in urine are preferred when evaluating thromboxane formation in vivo. 80 % of 11-dehydro TxB₂ excreted in urine is derived from platelets and 20 % from non-platelet sources [54,55].

1.4 PLATELETS AND ARTERIAL THROMBOSIS

Platelets are key cellular components of an arterial thrombus [15]. The rupture of a plaque exposes a highly thrombogenic surface and this initiates platelet adhesion and aggregation followed by the formation of thrombin and vasoconstriction caused by substances released from or present on the aggregating platelets. A major plaque rupture with extensive exposure of collagen and substances in the atheromatous core

may lead to immediate formation of an occlusive thrombus with sudden cardiac death or myocardial infarction (MI). A minor disruption may cause a non-occlusive thrombosis and promote slow progression of atherosclerosis [14,56].

Atherosclerotic plaques are classified into eight morphologically distinct categories among which two are called vulnerable plaques. They are often small, but have a high lipid content [3,57] which makes them soft and vulnerable to disruption with ulceration and subsequent thrombosis [3,58]. During high shear flow the lipid-rich core, abundant in cholesteryl esters, display the highest thrombogenicity in terms of platelet aggregating effects [59]. Lipid-lowering therapy may decrease this thrombogenicity [60].

Platelet activation by shear stress seems to be important in arterial thrombosis [61]. Shear-induced platelet aggregation is enhanced in patients with acute MI [62,63]. Moake et al showed that platelet activation in response to disordered shear stress depends on the presence of plasma vWF and the functional platelet receptor complexes GPIb-IX-V and GPIIb/IIIa [64]. At low shear stress (<12 dynes/cm²) fibrinogen seems to be the bridging ligand but at higher shear levels plasma or subendothelial vWF is the ligand mediating aggregation and platelet adhesion [65]. In stenosed arterial vessels there is high shear stress and the ligand receptor affinity is changed so vWF binds to both GPIb and GPIIb/IIIa [66]. ADP and adrenaline act synergistically with shear force, and inhibition of ADP counteracts shear induced platelet activation [65]. COX-inhibition by aspirin, on the other hand, has little effect on the initiation of aggregation by shear [63,65].

Inflammation and thrombosis are interrelated pathophysiological processes that involve multicellular activities [67]. Activated platelets increase leukocyte adhesion to the endothelium and promote leukocyte activation through the deposition of chemokines on the endothelium [68].

P-selectin is a membrane glycoprotein located in secretory α -granules in platelets and in endothelial cells [69]. P-selectin is rapidly redistributed to the plasma membrane upon cell activation [45]. P-selectin mediates interactions between platelets, leukocytes and endothelial cells [70] and stabilizes the initial interaction between GPIIb/IIIa and fibrinogen [71]. The main ligand for P-selectin is P-selectin Glycoprotein Ligand-1 (PSGL-1), a disulfide-linked homodimer consisting of two polypeptide chains which is constitutively expressed on most leukocytes [72]. PSGL-1 is also found on platelets and can mediate platelet-endothelial interactions [73]. The expression of P-selectin is transient and it is endocytosed [74] or proteolytically shed from the platelet surface into plasma. Soluble P-selectin has been proposed to be a reliable marker for in vivo platelet activation [75,76]. In addition, P-selectin may be present on circulating platelet microparticles formed during platelet activation.

CD40 ligand is a member of TNF- α family and is expressed by activated platelets [77] but was originally identified on activated T-cells. CD40L triggers an inflammatory response of endothelial cells [77], and stabilizes platelet rich thrombi. More than 95 % of the CD40L in whole blood is in platelets [78].

Platelet-leukocyte aggregates (PLAs) are formed both in vivo and in vitro [79,80] and represent an interface between inflammatory, thrombogenic and atherogenic responses. Granulocytes, lymphocytes and monocytes can all form PLAs [79,81]. Several ligand-receptor systems are involved in the formation of PLAs, including P-selectin and its

receptors. Increased PLA formation has been found in various diseases such as ischemic heart disease and diabetes mellitus [82-84].

1.5 EFFECTS OF EXERCISE AND MENTAL STRESS

Triggering of MI by heavy physical exertion and other various stressful conditions has been observed in several studies [13,56,85-87]. The risk is higher in sedentary individuals compared to individuals who are more physically active [88]. Physical exercise activates platelets more markedly than mental stress [89], but both mental and physical stress increase heart rate and blood pressure leading to increased myocardial oxygen demand and may cause plaque rupture [56]. During stress platelets may be activated by the sympathetic nervous system, the hypothalamic-pituitary-adrenal axis and serotonergic pathways [10,13,87,90]. During physical exercise platelets may be activated by α_2 -adrenoreceptors that are stimulated by catecholamines [91,92]. A prothrombotic state with procoagulant activation (increased thrombin generation, platelet hyperreactivity, and increased activity of coagulation factors) outweighing the concomitant activation of fibrinolysis was observed during high intensity physical activity [93]. On the other hand platelet activation can be suppressed by moderate exercise [13,94]. Of note, regular exercise training [93] or preceding warm-up exercise [95] reduces the platelet responsiveness to exercise. Individuals performing regular exercise exhibit lower baseline coagulation activity compared to sedentary individuals and this, together with the reduction of platelet responsiveness, is thought to be one explanation for the finding that regular physical activity reduces atherothrombotic risk in the long run [93].

1.6 PLATELETS AND PREECLAMPSIA

Normal pregnancy is associated with elevated levels of coagulation factors and increased platelet activation [96,97]. In patients with preeclampsia the hemostatic activity is further increased as reflected by increased thrombin generation, platelet activation and fibrin deposition in the placental vasculature [98]. Preeclampsia occurs in approximately 6 % of all pregnancies, and is one of the most common causes of mortality and morbidity in mothers and children in the Western world [25,99]. The disease process starts early in pregnancy but clinical signs of disease (high blood pressure and proteinuria) become apparent after 20 weeks of gestation [100]. Intrauterine growth retardation is often associated with preeclampsia, and is related to reduced uteroplacental blood flow [101,102]. The risk of developing preeclampsia is higher among women with diabetes mellitus and/or a previous history of preeclampsia, chronic hypertension, renal disease or multifetal gestation [103].

There is some evidence that platelets are involved in preeclampsia [104-106] and that the balance between the production of TxA_2 from platelets and prostacyclin (PGI_2) from endothelial cells is altered [107]. Endothelial dysfunction and a decrease in the prostacyclin/thromboxane ratio favours hypertension and may contribute to the reduction of uteroplacental blood flow seen in preeclampsia [108]. Patients with previous preeclampsia have an increased risk for developing hypertension and CVD [109] later in life, and large follow-up studies have found that there is an increased risk of cardiovascular death in women with previous preeclampsia, particularly when preterm delivery occurred [110,111].

1.7 ASSESSMENT OF PLATELET FUNCTION.

Assessing platelet function and effects of antiplatelet therapy is complicated since no single laboratory test has been shown to be optimal in determining the effect of a drug [20,112]. Platelets can be activated by several different mechanisms, and they interact with each other and with vascular cells and leukocytes. The relationships between surrogate endpoints reflecting platelet function and clinical events are a critical issue. Assessments of platelet function entail many methodological issues. Precautions should be taken to minimize platelet activation *in vitro*. It is, e.g., important with careful blood sampling (i.e. free flow and no stasis) and immediate processing of the sample [113]. The performance of a platelet function test will depend on the skill of the user and there is, e.g., poor standardisation between laboratories regarding how light transmittance aggregometry (LTA) is performed [114]. Different tests for aspirin sensitivity showed poor agreement between them [115].

Anticoagulants must be used and they may artificially modify platelet responses [116]. Sodium citrate is the most commonly used anticoagulant which acts by chelating extracellular calcium. It has been shown that platelet secretion and aggregation stimulated by various agonists is enhanced in low calcium media [117-119]. Low extracellular calcium levels will result in enhanced generation of platelet-derived TxA₂ and this causes the “second wave” aggregation after exposure to ADP and adrenaline; this phenomenon does not occur at physiological Ca²⁺ concentrations (1-2 mM) [120]. The selective thrombin inhibitor hirudin appears to be the most suitable anticoagulant for studies of platelet aggregation with normal extracellular calcium concentrations [116].

Most studies of platelet function are performed in platelet rich plasma (PRP), which requires centrifugation of the blood. This procedure may remove the largest and most active platelets but also red and white cells which are known to influence platelet behaviour [121]. In addition, in PRP platelet-platelet contact is facilitated which may increase platelet responsiveness in a non-physiological manner. A more physiological approach is therefore to study the platelets in whole blood [122].

1.7.1 Platelet aggregation

The most common *in vitro* technique is LTA, which is sometimes called Born aggregometry as it was originally described in the early 1960's by G Born [123]. This method measures changes in light transmittance in PRP following platelet aggregation. Using this method dose-response curves for aggregation induced by different agonists and effects of drugs can easily be investigated.

Platelet aggregation can also be measured in whole blood by impedance platelet aggregometry [124]. This method measures the deposition of platelets and platelet aggregates on electrodes in whole blood, i.e. in the presence of other blood cells, and may thus be considered to be more “physiological” than LTA.

Another method that assesses platelet aggregation in whole blood is filtragemetry *ex vivo*, as described by Hornstra [125]. Filtragemetry measures platelet aggregate formation (aggregability) in whole blood drawn directly from an antecubital vein and in the presence of physiological calcium levels. Our group has used this technique to study dynamic changes of platelet function *in vivo* and effects of various kinds of stress and stress hormones [12,89-92].

1.7.2 Flow cytometry

Flow cytometry is a powerful technique for platelet studies which requires only small amounts of blood and allows platelet activation to be studied in a physiological milieu. Flow cytometry detects individual cells/particles or cellular conjugates that pass a laser beam during a continuous flow of cells. To detect specific alterations on the surface of or in activated platelets fluorescently labelled monoclonal antibodies or fluorescent dyes are used. Fluorescence and scatter light signals are recorded by specific photodiodes. Mean fluorescence intensity (MFI) or % positive cells can be determined to measure the expression of antigen following binding of an antibody. The cell size and granularity are proportional to forward and side scatter of light, respectively.

1.7.3 Analysis of 11-dehydro-TxB₂ in urine and plasma

11-dehydro-TxB₂ is the major metabolite of TxA₂ in man [126,127]. The urinary excretion of 11-dehydro-TxB₂ appears to reflect phasic thromboxane generation and platelet activation in humans [126,128]. It has a longer half-life in plasma than 2,3-dinor-TxB₂ (45 vs 15 minutes) [49]. With regard to their urinary excretions, the fractional elimination of 11-dehydro TxB₂ was independent of the rate of TxB₂ infusion whereas the elimination of 2,3 dinor-TxB₂ was nonlinearly related to the dose of TxB₂ [128]. Efficient purification of human urine is needed when analyzing prostanoids by enzyme immunoassay (EIA) due to the large amounts of unrelated material in these fluids that interfere with the ligand-antibody binding in the assay. Even the “gold standard” method for analysis of 11-dehydro TxB₂, i.e. gas-chromatography-mass spectrometry (GC-MS), requires a good extraction procedure for reliable measurements [129].

1.8 ANTIPLATELET TREATMENT

1.8.1 Aspirin

Aspirin is an extensively evaluated antiplatelet drug which reduces cardiovascular events in various clinical settings by 19-32 % depending on the dosage [16]; in unstable coronary artery disease the protective effect of aspirin treatment is even greater [130]. Aspirin has also been used to prevent the development of preeclampsia. In a Cochrane review a 17 % reduction of the risk of developing preeclampsia by antiplatelet treatment was reported [131].

1.8.1.1 Mechanism of action

The antiplatelet effects of aspirin are considered to be mediated mainly by irreversible inhibition of platelet COX which blocks the synthesis of the strong platelet agonist TxA₂ [132]. As mentioned above COX occurs in two isoforms, COX-1 and COX-2, of which platelets under normal conditions mainly contain COX-1. Aspirin is 50-100 fold more potent in inhibiting platelet COX-1 than COX-2 [133]. Platelet COX-1 activity is irreversibly inhibited by acetylation of a serine residue (ser529), which prevents access of the substrate AA to the catalytic site of the enzyme [134], thus inhibiting the cyclooxygenase activity of the enzyme. The inhibitory effect lasts the life span of the platelet [135] because mature platelets are anucleate and therefore not able to regenerate COX. The effect of repeated daily low doses of aspirin is cumulative and a steady state with regard to platelet COX inhibition is reached within approximately 7

days [136]. After a single high dose of aspirin, platelet COX activity recovers by approximately 10 % per day as a function of platelet turn-over [137]. The recovery rate may, however, vary both within and between patients.

According to older studies, the relationship between thromboxane formation and platelet aggregation is non-linear and as little as < 5 % residual capacity to generate TxA_2 is enough to fully sustain thromboxane dependent platelet aggregation [138,139]. However, this concept has recently been challenged as Armstrong found a linear relationship between the degree of inhibition of platelet TxA_2 generation and TxA_2 mediated aggregation [140].

A high platelet turnover is associated with a larger population of circulating immature reticulated platelets with a higher granule content and residual megacaryocyte-derived mRNA. Reticulated platelets have a greater prothrombotic potential than smaller platelets [141]. In situations with high platelet turn-over newly formed platelets replenish platelet COX-1 more rapidly, thus allowing faster restoration of thromboxane synthesis by platelets not previously exposed to aspirin [142]. Recovery of serum TxB_2 was, e.g., found already 4 hours after ingestion of 650 mg aspirin in healthy volunteers [139]. COX-2 can be detected in patients with high platelet turnover and many circulating reticulated platelets, which might to some degree, explain aspirin-insensitive TxA_2 synthesis [143].

1.8.1.2 Pharmacokinetics

Aspirin is rapidly absorbed and inhibition of platelet function can be seen within 60 minutes. The plasma half-life of acetylsalicylic acid is 20 minutes, whereas that of salicylate is considerably longer [144]. At low dosages, first pass hepatic metabolism of aspirin limits the systemic exposure of the patients to COX inhibition and preserves the vasodilating and antiplatelet effects of endothelial PGI_2 [145]. Thus, low-dose aspirin acts presystemically in the portal circulation with regard to platelet inhibition [55,146].

1.8.1.3 Effective dosage and clinical efficacy of aspirin

Aspirin is an effective antithrombotic agent when used in doses ranging between 30-1500 mg/day. 75-150 mg daily appears to be the most effective dose [16]. There is no evidence that these low doses are less effective than high doses (i.e., 650-1500 mg/day) with respect to platelet inhibition, but lower doses produce fewer gastrointestinal side effects, and low doses may also have greater “antithrombotic” effects as the COX-2 mediated synthesis of PGI_2 in vascular endothelial cells is less affected [16,46,55].

Several studies have shown that aspirin effects are limited and that some patients at risk may not benefit from aspirin protection against cardiovascular events. This phenomenon is often named “aspirin resistance” and should not be confused with “treatment failure” which may be related to other disease mechanisms and poor compliance rather than “true” or “biochemical” aspirin resistance [19,22,147]. Aspirin has variable platelet inhibitory effects when assessed by different methods. Using 6 different tests 2.8-59.5 % of 201 patients in a study were judged not to have optimal antiplatelet effects during aspirin treatment [115]. The prevalence of “aspirin resistance” was highest using the Platelet Function Analyser PFA-100, a point-of-care method that activates platelets by shear stress and agonists (epinephrine and collagen) and lowest using LTA with AA (1.6 mM) as agonist.

With the use of “COX-specific” methods such as LTA+AA and measurements of platelet dependent thromboxane formation, the incidence of true “aspirin resistance” in compliant patients is probably as low as < 1 % [22,54]. Non-specific methods such as collagen- or ADP-induced platelet aggregation in PRP or urinary thromboxane metabolite excretion yield much higher frequencies of “aspirin resistance” [22,54,115,147,148]. In meta-analyses, however, patients with signs of “aspirin-resistance” had an approximately four-fold increased risk of developing cardiovascular events regardless of the specificity of the assay method used to assess platelet inhibition by aspirin [148,149]. Thus, both methods that selectively measure platelet COX inhibition and methods that assess platelet function more globally carry prognostic information, and which method(s) that may be suitable for clinical use by point-of-care testing is still debatable.

Poor compliance is a matter of great concern when responses to drug treatment are evaluated, and aspirin treatment is no exception. It is possible to assess aspirin compliance, e.g. through various biochemical assessments, but this is missing in the majority of studies assessing aspirin effects on platelet function [137]. In addition to compliance, the importance of hyperreactive platelets, and the time between measurements and the last intake of aspirin (which is seldom noted) have to be taken into consideration when evaluating “aspirin resistance” [19] .

Healthy women exhibit greater platelet aggregation in response to different agonists and have a greater urinary TxB₂ excretion compared to men before aspirin treatment [150]. The degree of inhibition by aspirin was significantly less in women when assessed using collagen and ADP. With a cutoff of >70 % inhibition of collagen-induced platelet aggregation, 30 % of women and 16 % of men were “aspirin resistant” [150].

As mentioned above, aspirin has also been used to prevent the development of preeclampsia. However, in the largest randomized controlled study of aspirin performed so far, the CLASP study, preventive treatment with aspirin 60 mg daily (usually after 12 weeks of pregnancy) compared to placebo only tended to reduce the incidence of proteinuric preeclampsia by 12 % (not significant) [25]. Reasons behind this relatively poor efficacy might be that the dosage of aspirin used was too low and/or that the treatment was initiated too late.

1.8.1.4 Other aspirin effects

There is some evidence that mechanisms unrelated to its ability to inactivate platelet COX-1 may contribute to the antithrombotic effects of aspirin. For example, inhibition of thrombin generation [151-153] and increased fibrin network permeability [154-156] which facilitates fibrinolysis [157] may contribute. Aspirin also decreased the maximum rate of FXIII cleavage and delayed FXIII activation by thrombin in a model of microvascular injury [158]. Reduced TF expression in human atherosclerotic plaques has been seen after aspirin treatment in smokers [159]. An interesting finding is that inhibition of thrombin generation by aspirin 75 mg/day was seen only in patients with total plasma cholesterol levels below 5,2 mmol/L [158].

1.8.2 Clopidogrel

The clinical efficacy of clopidogrel treatment is very well documented [17,20,160,161]. Today, treatment with thienopyridines including clopidogrel in

combination with aspirin is the “gold standard” for attenuation of platelet activation during acute coronary syndromes and in patients undergoing coronary stenting [6]. The CAPRIE study [162] showed a slightly greater reduction of vascular events with clopidogrel (75 mg/day) compared to aspirin (325 mg/day) in high risk patients with atherosclerosis. However, no comparison with the optimal dose of aspirin 75-150 mg/day [16] has ever been performed in a large scale setting. Combined therapy with aspirin and clopidogrel is indicated during and after an acute coronary syndrome and in the setting of coronary artery stenting following PCI. The duration of dual antiplatelet treatment is a matter of debate but it should only be given during limited periods of time due to the time course for the risk-benefit ratio [160,163]. The CHARISMA study randomized patients at high cardiovascular risk with pre-existing cardiovascular disease or risk factors to combined treatment with clopidogrel and aspirin or aspirin alone; the effects of combined treatment were less marked than in the CURE study and a significant increase of bleeding was seen [17,164].

1.8.2.1 Mechanism of action

Treatment with clopidogrel selectively and irreversibly inhibits the low affinity P2Y₁₂ receptor on platelets [165,166], resulting in non-competitive inhibition of ADP-induced platelet fibrinogen binding and platelet aggregation. The active metabolite of clopidogrel is highly labile and reacts as a thiol reagent with the ADP-receptor thus causing irreversible alterations of the receptor [167]. This is consistent with time-dependent cumulative inhibition of ADP-induced platelet aggregation upon repeated daily dosing without or with a low loading dose, and there is a slow recovery of platelet function after drug withdrawal [166]. Clopidogrel does not affect the P2Y₁ or P2X₁ purinergic receptors on the platelet surface. Clopidogrel treatment reduces the stability of platelet aggregates and may also inhibit platelet aggregation induced by low concentrations of collagen or thrombin. The latter most likely reflects blockade of the amplification of platelet responses to other agonists mediated by ADP released from activated platelets [137]. Clopidogrel may also attenuate PLA formation and reduce the plasma concentrations of CRP, soluble P-selectin and CD40L [168-170].

1.8.2.2 Pharmacology of clopidogrel

Clopidogrel is a prodrug that requires metabolism by the hepatic cytochrome P450 enzyme system in order to form the active metabolite and exhibit antiaggregatory effects. Clopidogrel is metabolized in two steps, first an oxidation to 2-oxoclopidogrel and then hydrolysis to the active metabolite [171-173]. SR26334, an inactive carboxylic acid derivative, is the principal circulating metabolite [174]. The plasma elimination half-life of the inactive metabolite is approximately 8 hours (h) [137], whereas the half-life of the active metabolite is only approximately 0,7 h [175]. The active metabolite of clopidogrel does not influence ADP-induced shape change [165]. P2Y₁- and P2Y₁₂ receptors act synergistically through different signalling pathways and therefore there is a limited effect of inhibiting only the P2Y₁₂ receptor by clopidogrel treatment [176].

1.8.2.3 Effective dosage and clinical efficacy of clopidogrel

There is a relationship between clopidogrel non-responsiveness and/or high on-treatment platelet reactivity and adverse clinical ischemic events [20,21]. In patients undergoing stenting for acute MI there was a 40 % increased 6-month risk of suffering

a recurrent cardiovascular event among patients in the highest quartile of ADP-induced aggregation during clopidogrel treatment, i.e. loading dose 300 mg and maintenance dose of 75 mg daily [177]. Increasing the dose of clopidogrel produces increased inhibition of ADP-induced platelet aggregation up to a plateau of inhibition of 50-60%, on average [137]. The initially used loading dose of 300 mg clopidogrel [163,177,178] has been replaced by 600 mg [179] which, when followed by 75 mg daily, produces rapid and sustained platelet inhibition. However, there are numerous reports of response variability and non-responsiveness to clopidogrel therapy [180,181]. It has been shown that non-responsiveness is dependent on the dose. Thus, a 600 mg loading dose reduced clopidogrel non-responsiveness to 8 % compared to 28-32 % after a 300 mg loading dose in patients undergoing elective PCI [182].

LTA is the most commonly used method to evaluate clopidogrel responsiveness and residual post-treatment P2Y₁₂ activity through measurements of ADP-induced platelet aggregation [180]. However, ADP also binds to P2Y₁ receptors and the effects of ADP on platelet function also reflect unblocked effect on ADP signalling through P2Y₁ [176]. Measurements of the vasodilator-stimulated phosphoprotein (VASP) phosphorylation by flow cytometry are specific with regard to signalling through P2Y₁₂ receptors and therefore more specific for the inhibitory effects of clopidogrel [180,183]. Approximately 20% of patients undergoing PCI were judged to be clopidogrel non-responders by various laboratory methods, and they exhibited an increased risk of worsened cardiovascular outcomes [184]. However measurement of responsiveness (absolute or relative changes in platelet aggregation compared to before treatment) may not be the optimal method to find patients at high risk due to the interindividual variability in baseline ADP-induced platelet aggregation [21]. The absolute level of platelet reactivity during treatment is proposed to be a better measure of thrombotic risk [21].

The mechanisms behind clopidogrel response variability and resistance are incompletely defined. Poor patient compliance is an important consideration and probably an underestimated cause of inadequate responses to clopidogrel [185]. Differences in intestinal absorption, differences in the conversion to the active metabolite by hepatic cytochrome P450, and platelet receptor polymorphism have been suggested to contribute to the response variability [175,185,186]. For example, CYP2C19, which is involved in the metabolism of clopidogrel to the intermediate metabolite, exhibits polymorphisms [180,187] and this and other isoenzymes may be a target for drug-drug interactions. There are several commonly prescribed competitive substrate drugs for CYP2C19, among which omeprazol and esomeprazole (PPI) have received most attention. Pharmacodynamic and pharmacokinetics studies suggest that concomitant use of clopidogrel and PPI reduces antiplatelet effects of clopidogrel using various platelet assays but effects on cardiovascular outcomes have not been established [188]. An interaction between CYP3A4 metabolized statins and clopidogrel has been proposed [189,190] but Malmström et al were unable to verify any importance of such a drug-interaction [191]. In summary, there is no consistent evidence that clopidogrel-drug interactions are of clinical importance [192].

2 AIMS OF THE STUDY

- To develop and optimize an enzyme immunoassay for analysis of 11-dehydro-TxB₂ in urine for measurements of thromboxane generation in vivo.
- To evaluate the dose- and time-dependence of the platelet inhibition effects of aspirin in order to better understand the potentials and limitations of treatment with this drug.
- To investigate the production of thromboxane in normal pregnancy and in pregnancy complicated by preeclampsia, as well as effects of aspirin treatment.
- To study the effect of clopidogrel treatment during platelet activation in vivo by exercise, in healthy volunteers and in aspirin treated patients with stable coronary artery disease (CAD).

3 PATIENTS AND METHODS

3.1 STUDY POPULATION

Urine samples were obtained from thirteen healthy volunteers and twenty-eight patients with CAD for the methodological work in study I. Urinary samples were also obtained from eight healthy subjects who were free from treatment with aspirin and other platelet inhibiting drugs before, and 12 hours after the ingestion of 500 mg aspirin in study I.

Thirty healthy male volunteers with easily accessible antecubital veins between 21-39 years of age were recruited among medical students for studies II and IV. One volunteer nearly fainted after exercise on the second occasion in paper IV. After the experiment he reported lack of sleep and malaise prior to this vaso-vagal episode and we therefore excluded him from the study.

Thirty-one male patients with stable angina pectoris, a pathological exercise stress test and CAD documented by coronary angiography, participated in study V. All patients were on the waiting list for elective coronary artery by pass grafting (CABG) at the Karolinska Hospital.

Twenty-eight pregnant women at high risk for developing preeclampsia (i.e., with a history of previous preeclampsia and/or intrauterine growth retardation, SLE or hypertension) and twenty-two healthy normotensive pregnant women participated in study III.

3.2 STUDY DESIGNS

3.2.1 Study II

The study was an open study with measurements at baseline (untreated) and during treatment with three different dosages of aspirin (fig 3). Measurements were performed at baseline and after aspirin treatment with 37.5 mg/day for 10 days and 320 mg/day for 7 days with a wash-out period of two weeks between low- and high-dose aspirin. Finally, they took a single dose of 640 mg aspirin 2 h after the last 320 mg dose. Blood sampling was performed before, 1.5 – 2 h, 6 h and 24 h after 37.5 mg, before and 1.5-2 h after 320 mg and finally 4 and 24 h after the last very high dose of aspirin (fig 3). Each subject brought his morning urine to the laboratory before the first blood sampling and when he returned the next day for 24 h sampling. Urine samples were also collected at 1 PM on days of experiment.

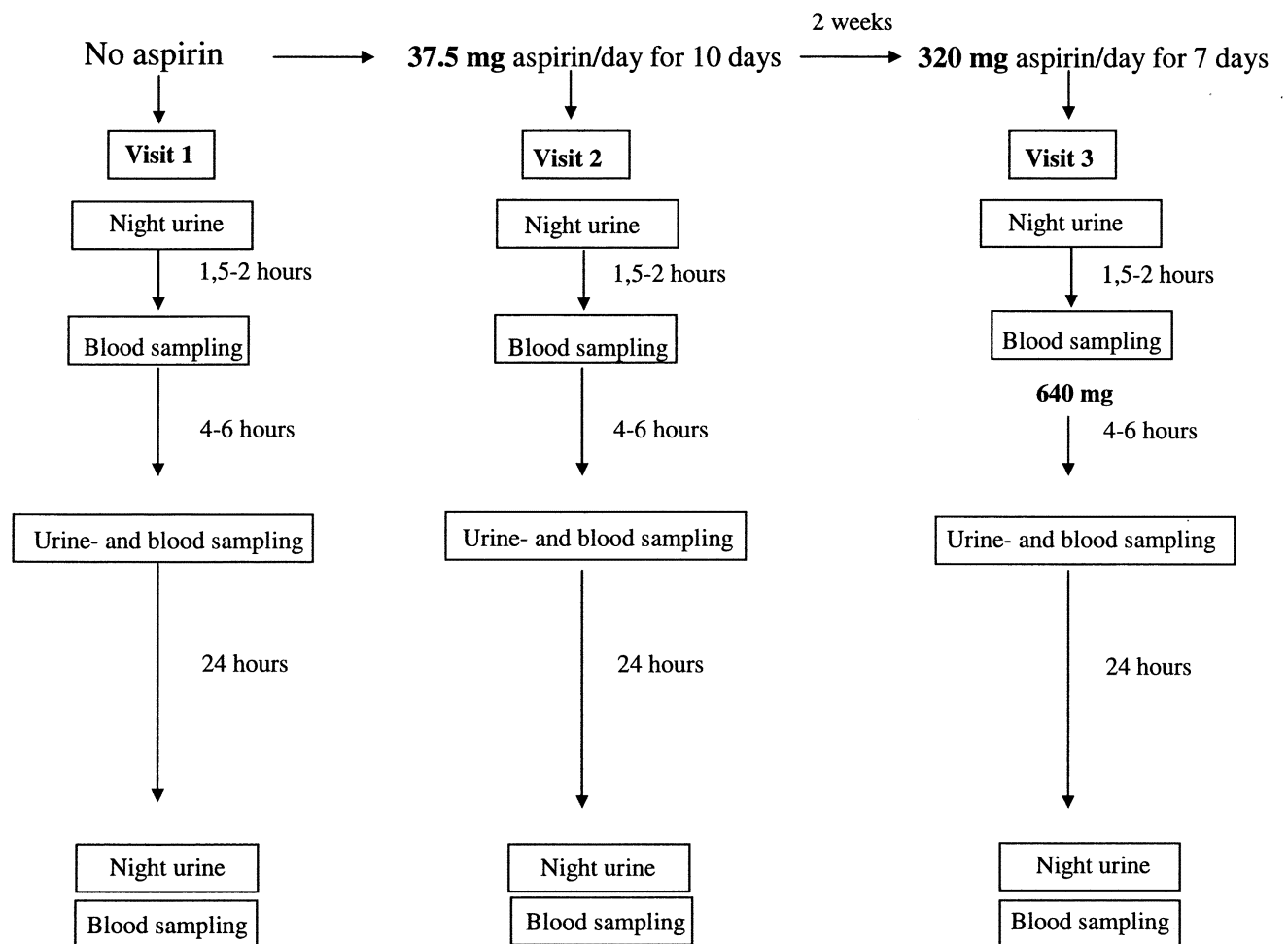


Figure 3: Design of study II

3.2.2 Study III

Aspirin treatment of patients with high risk of suffering preeclampsia commenced between gestational weeks 6-13 and was supposed to be stopped 2 weeks before delivery. Urine samples were collected before aspirin treatment, after two weeks of aspirin treatment, in pregnancy week 28-32, and 3-9 days post partum. Urine samples from healthy pregnant women (controls) were collected on four occasions: before gestational week 13, between gestational week 32-36, and 3-9 days and 3 months after delivery.

3.2.3 Study IV

The study was a randomized, open cross-over study comparing the effect of exercise without and with pretreatment with clopidogrel 75 mg/day for 7 days in healthy male volunteers. The volunteers sampled urine from the preceding night upon awakening. Another urine sample was collected 1 hour after termination of exercise. Blood samples and filrtragometry readings for measurement of platelet aggregability were performed before and after an exercise test which was terminated upon exhaustion.

3.2.4 Study V

The study was randomized, double-blind and placebo-controlled. All patients had ongoing treatment with aspirin (75-160 mg daily) during the entire study. After the first study visit the patients were randomized to treatment with either clopidogrel 75 mg or matching placebo once daily. The patients visited the laboratory before and after 14 days of study drug treatment. Blood samples were taken before and immediately after exercise. The exercise test was symptom limited and terminated upon severe chest pain, shortness of breath or fatigue, or other signs of severe myocardial ischemia (Electrocardiographic recordings (ECG) changes), falling blood pressure or severe arrhythmias). Continuous ambulatory ECG monitoring (48 h) was performed at inclusion and after two weeks of study drug treatment in order to evaluate treatment effects on ambulatory myocardial ischemia.

3.3 BLOOD SAMPLING

All subjects had antecubital veins allowing technically good sampling. Blood sampling for baseline or pre-exercise measurements was performed after 30 minutes of rest in the supine position. Upon termination of exercise blood sampling was repeated immediately. Healthy volunteers and patients were instructed to refrain from caffeine and tobacco ≥ 12 h before experiments. They were also instructed not to take any platelet inhibiting drugs during 14 days preceding sampling. In study V all patients received chronic aspirin treatment 75-160 mg once daily (see above).

3.3.1 Anticoagulants

We used hirudinized blood in study II to avoid artifactual enhancement of the TxA₂-dependence of platelet aggregation (and thus the efficacy of aspirin) which is seen when citrate is used as anticoagulant and extracellular calcium levels are low [116,118,120].

3.4 PLATELET FUNCTION TESTS

3.4.1 Bleeding time

Standardized transverse incisions were made on the lateral volar side of the forearm, at a constant venous pressure of 40 mmHg, using a disposable device (Surgicutt II, Ortho Diagnostics, Raritan, NJ, USA). Repeated incisions were made 10 mm distally to the previous one to avoid influences of previous measurements on the same occasion. Total bleeding time was measured by collecting blood on filter papers at 15 s intervals according to a previously described technique [193,194]. In our laboratory the intra-individual coefficient of variation for total bleeding time is 14 % [194].

3.4.2 Filtragometry

Filtragometry monitors platelet aggregate formation in whole blood ex vivo [125]. Blood is drawn continuously from an antecubital vein and anticoagulated by heparin before passing through a nickel filter with a pore size of 20 μ m. The apparatus may contribute to platelet aggregate formation (via contact with the siliconized tubing system and/or through shear forces when blood passes the filter) but this contribution is identical in all measurements. The time in seconds (aggregation time, t_A) taken to reach the predetermined degree of filter occlusion is inversely related to platelet aggregability in vivo [12,125,195]. A rapid filter occlusion with a low t_A value indicates high platelet aggregability. The coefficient of variation for between-day variability of two resting measurements is 7.8 % for log t_A [196].

3.4.3 Whole blood aggregometry

Platelet aggregation in whole-blood was studied in papers II and V using a four channel impedance aggregometer (Chrono-log model 570-VS four Sample; Chronolog Corp, Haverton PA USA). The blood was anticoagulated with recombinant hirudin (in paper II) or citrate (in paper V) and diluted 1:1 with physiological saline. Samples were preincubated at 37° C for 5 minutes, after which agonists were added. Agonists used were collagen type 1 (Horm Nycomed Arzneimittel, Munich, Germany) at final concentrations of 1, 3 and 5 μ g/mL (paper II) or 1 and 5 μ g/mL (paper V), and AA dissolved in ethanol (Sigma Chemical Co, St Louis, MO, USA) at final concentrations of 0.2, 0.5 and 1 mM (paper II) and 0.5 mM (paper V). The amplitude of aggregation was measured after 8 minutes.

3.4.4 Aggregometry in platelet rich plasma (PRP)

A four-channel platelet aggregation profiler (PAP-4, Bio-Data Corporation, Hatboro, PA, USA) was used in paper II to study platelet aggregation in PRP. ADP was diluted in Tris buffer and the EC₅₀ for ADP (i.e., the concentration required for half-maximal aggregation) was determined by a dose-response procedure in which the extent of aggregation after 4 minutes was measured [9]. Platelet COX inhibiting effects of aspirin treatment were determined as the ability of AA (final concentrations 0.5 and 1 mM) to induce aggregation in PRP.

3.4.5 Flow cytometric analysis

Five μL citrated whole blood was added to 45 μL of Hepes buffered saline (to minimize in vitro aggregate formation) within 3 min of blood sampling. The tubes contained appropriately diluted antibodies and agonists for detection of platelet P-selectin expression, leukocyte CD11b expression or platelet-leukocyte conjugates. After incubation for 20 minutes samples were further diluted and fixed with 0.5 % formaldehyde in saline before analysis in an EPICS XL-MCL flow cytometer (Coulter Corp, Hialeah FL). Flow cytometric analyses in papers IV and V were performed as described previously [195,197-199].

3.4.5.1 Single platelet analysis

Platelets were identified with the FITC-conjugated anti-CD42a (GPIX) monoclonal antibody Beb1 (Becton Dickinson, San Jose, CA, USA). Platelet P-selectin expression was identified with the RPE- (R-phycoerythrin) conjugated anti-P-selectin monoclonal antibody AC1.2 (Becton Dickinson). Platelets were first gated according to their characteristic light scattering signals, and then confirmed by FITC-CD42a staining (>99% positive). The gated cells (>99% platelets) were subsequently subjected to single colour analysis of RPE-CD62P fluorescence to obtain the percentage of P-selectin positive cells in the platelet population [195,197]. Agonists used for in vitro stimulation were ADP and human α -thrombin (fibrinogen polymerisation was prevented by adding the peptide GPRP when thrombin was used).

3.4.5.2 Platelet leukocyte aggregates (PLA)

Leukocytes were identified with the RPE-conjugated anti-CD45 monoclonal antibody J33 (Immunotech, Marseille, France), and gated as lymphocytes, monocytes and neutrophils according to CD45 expression and light scattering characteristics [198]. Total leukocytes and the different subsets were then subjected to two colour analysis (RPE-CD45 vs FITC-CD42a) to discriminate platelet-free and platelet-coupled leukocytes. Platelet leukocyte aggregates are reported as percentages and counts of platelet-bound total leukocytes (PLA), neutrophils, monocytes and lymphocytes.

3.4.5.3 Leukocyte CD11b expression

Leukocyte CD11b expression was analysed as a marker of leukocyte activation. Mean fluorescence intensities of FITC-CD11b staining among total leukocytes, granulocytes, lymphocytes and monocytes were reported [199]. Agonists used for in vitro stimulation were ADP and fMLP (N-formyl-methionyl-leucyl-phenylalanine).

3.4.6 Platelet function analyser (PFA-100)

The PFA-100 analyzer (Dade Behring, Germany) was used for measurements of platelet-related primary haemostasis capacity in citrate-anticoagulated whole blood under high shear conditions [200]. Closure time (CT) is the time needed to form a platelet plug occluding a small aperture in a collagen/epinephrine or collagen/ADP coated membrane. In the present project only the aspirin sensitive collagen/epinephrine coated membranes were used.

3.4.7 Thromboxane related measurements

Urinary 11-dehydro-TxB₂ was determined by EIA using commercially available reagents (Cayman Chemicals, Ann Arbor MI USA) and a sample work-up procedure described and validated in paper I. Importantly, 11-dehydro-TxB₂ occurs in two forms in a pH-dependent equilibrium. At basic pH it is converted to an open ring structure with a carboxyl group at C-11 which is a strong antigenic determinant influencing the affinity and specificity of antisera [201]. Thus, two ml of centrifuged urine was diluted 1:2 with 63 mM ammonium bicarbonate buffer pH 8.6 and was incubated for 3 hours to convert 11-dehydro-TxB₂ to its open ring form before extraction with Bond-Elute Certify-II columns (Varian, Harbour City, CA, USA). The analyte was eluted with 2% formic acid in ethanol. The eluate was evaporated in a vacuum centrifuge, resuspended in buffer (pH 8.6), and incubated for 6 hours before analysis. This method was also adapted for measurements in plasma in paper IV.

Serum TxB₂ was determined using commercially available kits for measurements of TxB₂ (Cayman Chemicals). Serum was produced by incubation of non-anticoagulated whole blood samples at 37°C for 1 hour, followed by centrifugation. Sample preparation and analysis were according to instructions from the manufacturer.

3.4.8 Urinary 2,3 dinor 6-keto prostaglandin F_{1α}

Analysis of the 2, 3-dinor-6-keto-prostaglandin F_{1α} metabolite of prostacyclin (PGI-M) in urine seems to be the most accurate method for assessment of systemic (extrarenal) prostacyclin biosynthesis [202]. Urinary PGI-M was determined in paper II by LC/MS-MS after solid phase extraction, liquid-liquid extraction and derivatization with methoxyamine (Rooney C, unpublished).

3.4.9 Soluble markers in plasma in paper IV and V

Thrombin generation was measured as prothrombin fragment 1+2 in plasma prepared from whole blood samples anticoagulated with 3.8 % citrate (Enzygnost F1+2; Behring Diagnostics, Marburg, Germany).

Blood samples for analyses of vWF, Il-6 and elastase in plasma were anticoagulated with 3.8 % citrate and centrifuged 10 min at 1400 x g at 4 °C. EIAs were used to determine vWF (Asserachrom, Diagnostica Stago, France), Il-6 (R&D Systems, Abingdon, UK) and elastase (DPC Biermann GmbH, Bad Nauheim, Germany). Soluble CD40L was determined by EIA (kit) in serum in study V and in EDTA plasma in study IV. Serum C-reactive protein (CRP) was determined by a high-sensitive nephelometric assay (Cardiophase hsCRP, Dade Behring).

3.5 STATISTICAL ANALYSIS

Descriptive statistics are presented as mean values ± SEM. Normally distributed variables were compared with paired t-tests. Effects of treatment were analyzed by 2-factor repeated measures ANOVA for overall effects. Skewed data were log transformed before analysis by ANOVA (papers IV and V). In paper I bleeding time data were compared with the Wilcoxon test and with Spearman's rank correlation. Statistica version 5.5 was used for calculations; p<0.05 was considered significant.

3.6 ETHICAL CONSIDERATIONS

Studies II and III were approved by the local Ethics Committee of the Karolinska Hospital. Studies IV and V were approved by the Ethics Committee of the Karolinska Institute and by the Swedish Medical Products Agency. Informed consent was obtained from all subjects. The urine samples used in study I were unidentified, and a gift from our co-author, Professor Desmond Fitzgerald, Dublin, for methodological studies and had been obtained with permissions and consent according to Irish regulations.

4 RESULTS AND DISCUSSION

4.1 OPTIMIZATION OF AN ENZYME IMMUNOASSAY FOR 11-DEHYDRO-THROMBOXANE B₂ IN URINE: COMPARISON WITH GC-MS. (PAPER I)

In this study we described an improved immunoassay procedure for 11-dehydro-TxB₂ in urine based on a one-step solid phase extraction using Bond-Elut Certify-II columns followed by EIA using commercially available reagents. A key feature of the assay was to keep 11-dehydro-TxB₂ in its open ring form with a carboxyl group at C-11 (a strong antigenic determinant) during the entire assay procedure by equilibrating at pH 8.6. This improved the performances of both the sample work-up and analysis steps. The detection limit of the assay (conservatively defined as the IC₈₀) decreased from 10±6 pg/mL with EIA-buffer pH 7.4, to 6±2 pg/mL with an EIA-Ambic buffer at pH 8.6.

Efficient purification of human urine is needed when analyzing prostanoids by EIA due to the large amounts of unrelated material that may interfere with the ligand-antibody binding in the assay. The Bond-Elut Certify-II columns contain mixed phase material with lipophilic and anion exchange parts. The open ring form of 11-dehydro-TxB₂ is retained in the column by ionic binding during washing with methanol. Elution of 11-dehydro-TxB₂ from the Certify-II columns was accomplished using formic acid in methanol as ion-changer. This extraction procedure resulted in a recovery of 83 % (95% confidence interval 74-92 %) in 11 urine samples spiked with 500 pg/mL 11-dehydro-TxB₂.

Intra- and interassay coefficients of variation of the final assay were 3 and 13.8 %, respectively. Comparison with GC-MS showed excellent agreement between the two methods ($r^2=0.94$, $p<0.0001$). Samples with high levels (>10 000 pg/mL) were overestimated by EIA. This is to be expected as specificity problems with an immunoassay procedure will increase when the amounts of prostanoids in urine are high. Overestimation by the EIA method at the highest levels may be due to the presence of other thromboxane metabolites with similar structural features (e.g. 11-dehydro-2,3 dinor-TxB₂) which is also abundant in urine and can be expected to behave similarly as 11-dehydro-TxB₂ in the extraction procedure. The administration of 500 mg aspirin decreased the urinary excretion of 11-dehydro-TxB₂ by 77±13 % in healthy volunteers, in agreement with prior results using GC-MS [202]. GC-MS methods incorporating an ideal internal standard are the most accurate when analysing 11-dehydro TxB₂ [129,203] but may be unsuitable for analysis of large numbers of samples due to limited analysis capacity and high costs.

In summary, we described a simple work-up procedure which, when combined with a modified commercially available EIA for 11-dehydro-TxB₂, provides results that agree well with those obtained by the gold standard method, GC-MS. Raising the pH to 8.6 results in dependable analyte behaviour during solid phase extraction, and also increases the sensitivity of the assay.

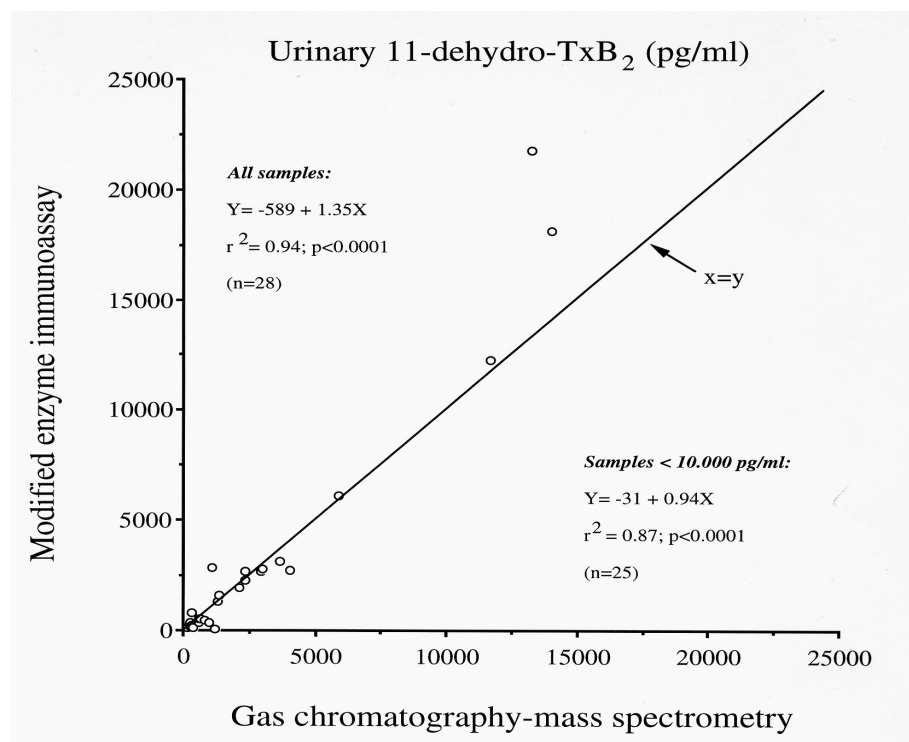


Figure 4: Comparison of concentrations of 11-dehydro-TxB₂ obtained by GC-MS and the modified EIA described presently in 28 samples with a wide range of 11-dehydro-TxB₂ concentrations. The line of identity is indicated. Equations and correlation coefficients (r^2) are indicated for all samples and for samples with <10 000 pg/mL 11-dehydro-TxB₂ by GC-MS.

4.2 DOSE- AND TIME-DEPENDENT ANTIPLATELET EFFECTS OF ASPIRIN. (PAPER II)

Fifteen male healthy volunteers ingested different doses of aspirin (37.5 mg during 2 weeks followed by 320 mg during 7 days, and, finally, a single dose of 640 mg 2 h after the last 320 mg dose, i.e., totally 960 mg on the last day) in an open cross-over study to evaluate the dose- and time-dependence of antiplatelets effects of aspirin.

Platelet COX-inhibition was nearly complete already with 37.5 mg aspirin daily as evidenced by >98 % suppression of serum TxB₂ and almost abolished AA induced aggregation in PRP 2-6 h after dosing. The platelet sensitivity to ADP decreased after 37.5 mg aspirin as the EC₅₀ for ADP increased by 65±11 % ($p < 0.01$) but this effect was less pronounced (not significant) after higher doses of aspirin.

Bleeding time was similarly prolonged by all doses of aspirin. Eight of the 15 investigated subjects had <60% increments of bleeding time after 37.5 mg aspirin and similarly low increments after 320 mg. There was a significant correlation between closure time (measured by PFA-100; collagen/epinephrine cartridge) and bleeding time after aspirin ($r=0.78$; $p < 0.01$, $n=6$).

The inhibition of AA-induced platelet aggregation in whole blood was time and dose-dependent during aspirin treatment. The inhibitory effects of low dose aspirin (37.5 mg daily) were significantly attenuated after 24 h compared to 6 h ($p < 0.01$). 24 h after

high-dose aspirin (320 mg daily) the aggregatory response to 1 mM AA was almost normalized in whole blood.

Collagen-induced (1 µg/mL) platelet aggregation in whole blood with normal extracellular calcium levels was inhibited <40% at all dosages. Higher collagen concentrations (3 and 5 µg/mL) yielded nearly maximal platelet aggregation in hirudinized whole blood despite treatment with aspirin (all dosages).

The nocturnal excretion of 11-dehydro-TxB₂ was reduced by 74.0±2.0 % (p<0.001) after 37.5 mg aspirin. Four hours after 960 mg aspirin the excretion of 11-dehydro-TxB₂ was reduced by 83.9±2.8 % (p<0.001 vs baseline and 37.5 mg), but there was a significant recovery after <24 hours to 78.5±1.4 % inhibition (p<0.001).

Aspirin treatment reduced the urinary excretion of PGI-M by approximately 25% already with the lowest aspirin dose. There was a significant recovery of the prostacyclin metabolite excretion <24 hours after 960 mg aspirin. We found no relationship between changes in the excretion of PGI-M and any platelet function variable.

As shown in Figure 6, the parameter most sensitive to inhibition by aspirin was AA-induced platelet aggregation in PRP, followed by serum TxB₂. The urinary excretion of 11-dehydro-TxB₂ was less markedly, but dose-dependently reduced by aspirin. Inhibition of collagen induced aggregation in hirudinized whole blood was also found with the lowest collagen concentration tested (1 µg/mL).

The effectiveness of aspirin is thus highly dependent on the method used to evaluate its antiplatelet effect. Anticoagulation by hirudin resulted in limited inhibition by aspirin of collagen and AA-induced platelet aggregation in whole blood. AA-induced platelet aggregation occurred even after 960 mg aspirin if the AA-concentration was high.

A problem with in vitro studies of platelet function in PRP is that centrifugation of blood may remove the largest and most active platelets along with the red and white blood cells which are known to influence platelet behaviour [204-206]. The less pronounced antiaggregatory effect of aspirin treatment in whole blood compared to PRP may reflect erythrocyte enhancement, extraplatelet sources of PGH₂ and/or transcellular formation of TxA₂ despite efficient inhibition of COX in platelets. Nucleated cells are capable of regenerating the COX-1 enzyme after a couple of hours and provide PGH₂ to platelets to bypass the inhibition of platelet COX-1 [207].

We found less pronounced inhibition of 11-dehydro-TxB₂ excretion compared to serum TxB₂ and partial recovery of 11-dehydro-TxB₂ excretion at the end of the dosing interval indicating that aspirin insensitive (presumably non-platelet) thromboxane synthesis had occurred in vivo.

In conclusion aspirin provides only weak inhibition of platelet aggregation in whole blood with normal calcium levels despite effective platelet COX inhibition and there is a recovery of platelet function in whole blood with a normal 24 h dosing interval in healthy volunteers.

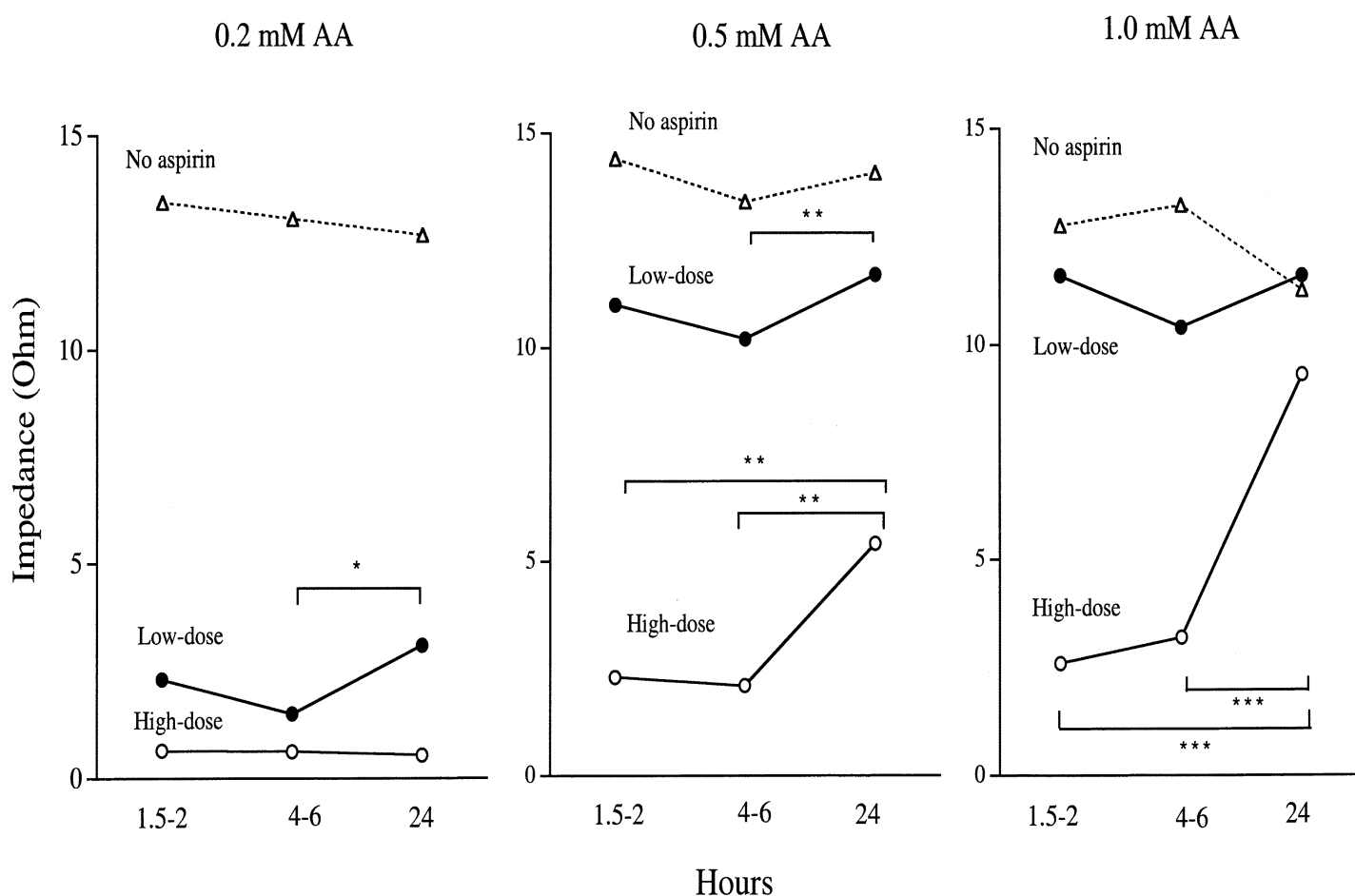


Figure 5: Comparison of AA-induced aggregation (final concentrations 0.2, 0.5 and 1.0 mM) in whole blood anticoagulated with hirudin after different doses of aspirin. Blood samples were taken 1.5-2, 4-6 and 24 hours after latest ingested dose. Low-dose is 37.5 mg/day for 10 days (filled symbols). High-dose is 320 mg/day for 7 days and a single dose of 640 mg taken immediately after the 1.5-2 hour sample (open symbols).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by paired t-tests.

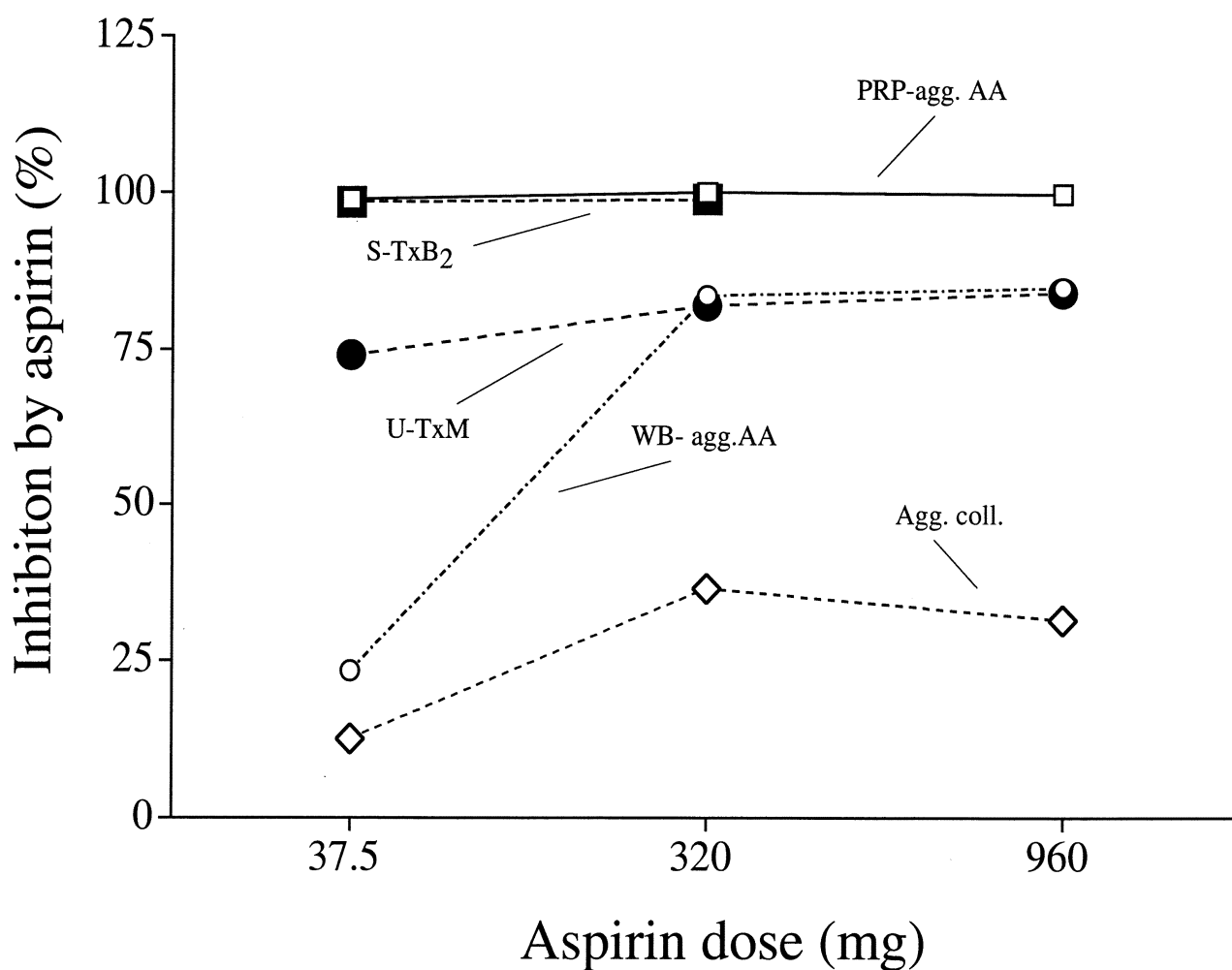


Figure 6: Comparison of platelet inhibition afforded by aspirin at different doses (37.5-960 mg) using different platelet function assays. Illustrated in the graph are: platelet aggregation in whole blood (WB) using arachidonic acid (AA; 0,5 mM) or collagen (Coll; 1 µg/mL); platelet aggregation in platelet rich plasma (PRP) using AA (1,0 mM) as agonists; serum (S) TxB₂; and 11-dehydro thromboxane B₂ (U-TxM) metabolite excretion in urine.

4.3 THROMBOXANE METABOLITE EXCRETION DURING PREGNANCY – INFLUENCE OF PREECLAMPSIA AND ASPIRIN TREATMENT. (PAPER III)

The urinary excretion of 11-dehydro-TxB₂ increased with the duration of normal pregnancy (fig.7). Three months after delivery the excretion was decreased to lower values than those found early in pregnancy.

The urinary excretion of 11 -dehydro-TxB₂ was significantly higher in patients at high risk of developing preeclampsia compared to the control group (129±12 vs 90±12 ng/mmol creatinine; p<0.05) before gestational week 13. These findings are in agreement with the postulated role for thromboxane in preeclampsia [208].

Aspirin treatment suppressed 11-dehydro-TxB₂ excretion to the same relative extent (by approximately 80 %) in pregnant women at high risk of suffering preeclampsia as previously found with the same methodology in healthy volunteers (Paper 1,II), and by Benigni et al in pregnancies at risk for pregnancy-induced hypertension [209]. Four of the 28 high-risk patients developed preeclampsia with increases in blood pressure and proteinuria. These patients had a higher excretion of 11-dehydro-TxB₂ before gestational week 13 than patients without complications (202±38 vs 117±10 ng/mmol creatinine p<0.01). The degree of inhibition by aspirin was at least as great in patients developing preeclampsia.

Our study thus shows increased 11-dehydro-TxB₂ excretion already before gestational week 13 in patients at high risk for preeclampsia compared to healthy pregnant women. These data imply that the elevation of thromboxane production, and the imbalance between thromboxane and prostacyclin which has been found in women with a high risk of suffering preeclampsia or actually manifesting the disease [210-212], commences very early in pregnancy. Thus, aspirin treatment should probably be started very early to increase the chance of influencing the development of preeclampsia by reducing platelet-dependent thromboxane formation.

Non-platelet dependent thromboxane formation may also be of importance in preeclampsia. Walsh et al [208] showed that trophoblast cells in preeclamptic placentas produced 7 times as much thromboxane as prostacyclin. Of interest is that trophoblast cells, in contrast to platelets, can regenerate COX-1. Furthermore, the trophoblast cells may not be exposed to effective concentrations of aspirin during low-dose treatment due to the efficient first-pass metabolism of aspirin noted above.

In summary, the present results indicate that 11-dehydro-TxB₂ excretion increases throughout normal pregnancy with the highest levels 3-7 days postpartum. Patients at high risk of suffering preeclampsia have an increased 11-dehydro-TxB₂ excretion compared to healthy pregnant women already before gestational week 13. Low-dose aspirin treatment (75 mg once daily) effectively inhibits platelet-dependent thromboxane production early in pregnancy, but the 11-dehydro-TxB₂ excretion increases later in pregnancy. These data imply that the modest effects of aspirin seen in preeclampsia prevention trials may be related to late initiation of therapy. The use of very low doses of aspirin (< 75 mg daily) may have contributed to the findings in trials.

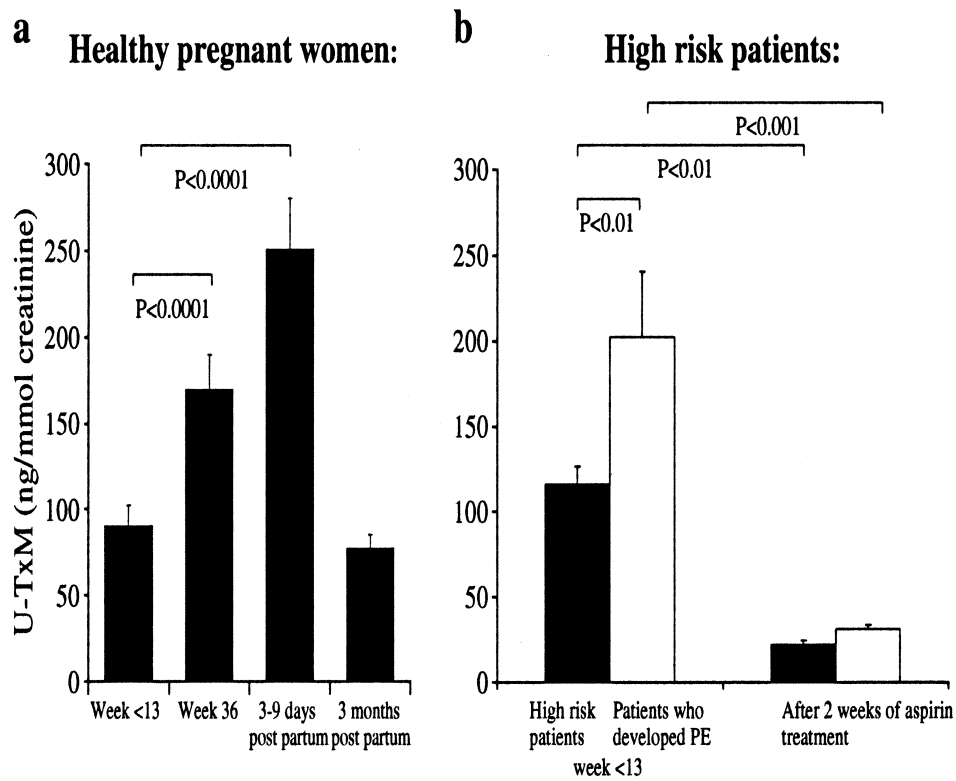


Figure 7: Left panel: Urinary excretion of 11-dehydro-thromboxane B₂ (U-TxM) in 22 healthy pregnant women during and after pregnancy. Right panel: Urinary excretion of TxM before and after two weeks of aspirin treatment in 24 patients at high risk for preeclampsia (filled columns) and 4 patients who actually developed preeclampsia (open columns). Mean values and SEM. P-values were obtained with paired t-tests.

4.4 PROTHROMBOTIC RESPONSES TO EXERCISE ARE LITTLE INFLUENCED BY CLOPIDOGREL TREATMENT

In study IV 15 healthy volunteers performed exhaustive exercise without and with clopidogrel pretreatment (75 mg/day; 7 days). The workload achieved in the exercise test was approximately 260 W on both occasions.

Exercise evoked platelet activation in vivo as reflected by increased circulating platelet-platelet aggregates (PPAs) ($p < 0.001$ by ANOVA), shortened filtragemetry readings ($p < 0.001$), increased P-selectin expressing single platelets ($p < 0.001$), and elevated levels of 11-dehydro-TxB₂ in plasma ($p < 0.001$). Exercise also enhanced the platelet responsiveness to ADP or thrombin in vitro ($p < 0.001$ for both by ANOVA). These effects of exercise are in agreement with previous results [195].

Clopidogrel treatment effectively inhibited platelet ADP-receptors as the P-selectin expression response to 10^{-5} M ADP in vitro was reduced by 72 ± 2.7 % (range 54-85 %), indicating good compliance and efficient blockade of P2Y₁₂ receptors. Clopidogrel treatment also attenuated the responses to thrombin but did not significantly attenuate the enhancing effect of exercise ($p = 0.20$ with ADP and $p = 0.19$ with thrombin as platelet agonists). Attenuation of agonist-stimulated platelet activation in vitro by clopidogrel may reflect positive feed-back by platelet-released ADP in response to submaximal stimulation by other platelet agonists [137,168,213]. This may have implications in the vicinity of the locally formed thrombi, even though it does not seem to influence the function of circulating platelets.

Clopidogrel treatment reduced platelet aggregability assessed by filtragemetry ($p < 0.05$) both at rest and following exercise but there was no significant effect of clopidogrel treatment on the enhancement of platelet reactivity to agonist stimulation by exercise ($P = 0.53$ by two-factor repeated measures ANOVA). The prolongation of filtragemetry readings by clopidogrel treatment at rest suggests that such measurements reflect an ADP-dependent component in platelet activation.

Exercise enhanced thromboxane formation as evidenced by measurements of 11-dehydro-TxB₂ in plasma, whereas urinary measurements could not detect any exercise effect. Urinary measurements are thus less sensitive than measurements in plasma when assessing responses to short-lasting stimuli in vivo. The elevation of 11-dehydro-TxB₂ in plasma probably reflects platelet activation during exercise, as most of the thromboxane metabolite emanates from platelets [202]. However, a small contribution from other cells such as monocytes cannot be ruled out.

Exercise increased circulating PLAs mainly due to leukocytosis, as the percentages of PLAs among leukocytes changed little. Plasma elastase was markedly elevated by exercise ($p < 0.001$ by ANOVA) indicating increased granulocyte secretion but was not influenced by clopidogrel treatment. Clopidogrel treatment did not influence the basal CD11b expression either before or after exercise among total leukocytes, neutrophils or monocytes, but attenuated responses of total leukocytes and neutrophils to ADP ($p < 0.05$). Monocyte CD11b expression also increased with ADP stimulation but this response was not attenuated by clopidogrel treatment. Clopidogrel treatment did not influence soluble CD40L levels either at rest or after exercise.

Clopidogrel treatment did not influence circulating PLAs either at rest or after exercise but did attenuate the responses to in vitro stimulation by both ADP and submaximal thrombin concentrations with regard to platelet-conjugated leukocytes and their

subpopulations. Platelet-lymphocyte aggregate responses to ADP stimulation in vitro were, however, slightly enhanced ($P<0.05$) by exercise, and this enhancement was attenuated by clopidogrel treatment ($P<0.05$) suggesting that platelet activation and ADP released from activated platelets influence agonist-stimulated PLA formation in vitro. These findings may have implications for the development of atherosclerosis since platelet conjugation may enhance the recruitment of leukocytes into the (dysfunctional) arterial wall [14,214].

In summary, strenuous exercise evoked multicellular activation vivo and promoted a prothrombotic state, but clopidogrel treatment did not attenuate the platelet response to exercise. The hypothesis that $P2Y_{12}$ -receptor dependent mechanisms are of importance for exercise induced activation of circulating platelets (perhaps due to increased shear stress involving ADP-dependent mechanisms [61]) was not supported in this study.

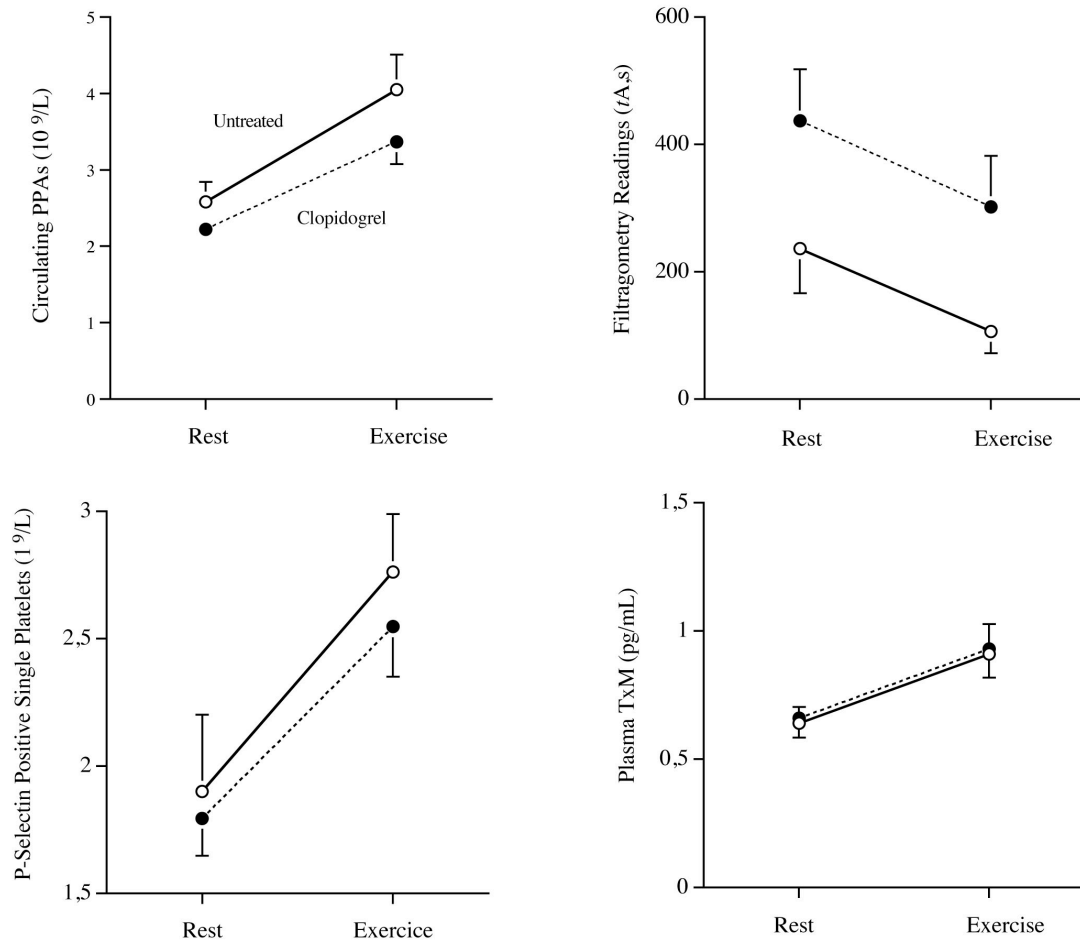


Figure 8: Exercise evoked platelet activation in vivo, as reflected by increased circulating platelet-platelet aggregates (PPAs) ($p < 0.001$ by ANOVA), shortened filtragometry readings (inversely reflecting platelet aggregability) ($p < 0.001$), increased P-selectin expressing single platelets ($p < 0.001$), and elevated levels of 11-dehydrothromboxane B_2 (TxM) in plasma ($p < 0.001$). Clopidogrel treatment (solid symbols and dashed lines) reduced platelet aggregability assessed by filtragometry ($p < 0.05$), but did not significantly attenuate any of these indices of platelet activation by exercise, as analyzed by two-factor repeated measures ANOVA.

4.5 EFFECT OF CLOPIDOGREL TREATMENT ON STRESS-INDUCED PLATELET ACTIVATION AND MYOCARDIAL ISCHEMIA IN ASPIRIN-TREATED PATIENTS WITH STABLE CORONARY ARTERY DISEASE.

In study V we investigated if the addition of clopidogrel treatment to ongoing aspirin treatment could attenuate exercise-induced platelet activation and reduce ambulatory and/or exercise-induced myocardial ischemia in 31 patients with CAD. The vast majority of patients in both randomized treatment groups had three-vessel disease. We had planned to include 44 patients but changes in the health care system in Stockholm abolished the waiting list for CABG surgery from which the patients were recruited and

made it impossible to include more than 31 patients as we considered it unethical to delay surgery for participation in the study.

The workloads achieved and the heart rate responses to exercise were slightly greater, and the signs of ischemia during exercise were slightly less pronounced in the placebo group compared to the clopidogrel group, both at baseline and during treatment.

At rest, clopidogrel treatment reduced the platelet P-selectin expression induced by 10 μ M ADP in vitro by 22-87% (mean 64%) and attenuated the responses to thrombin stimulation in vitro by approximately 35%. Clopidogrel treatment also attenuated collagen-induced platelet aggregation (1 μ g/ml) by $40\pm 10\%$ ($p<0.05$). Circulating PPAs reflecting platelet micro-aggregation in vivo were uninfluenced by clopidogrel treatment at rest. Again, attenuation of responses to other agonists than ADP (i.e., collagen and thrombin) in vitro reflects the importance of positive feed-back by platelet-released ADP [137,168].

Exercise enhanced the platelet responsiveness to agonist stimulation when assessed as the percentage of platelets expressing P-selectin, but there was no significant attenuation of the exercise effect by clopidogrel compared to placebo treatment with either agonist. The relative inhibition of ADP-induced platelet activation was similar at rest and following exercise indicating that the treatment may protect against thrombotic event also during stress caused by heavy exertion.

Platelet-conjugated monocytes were reduced by clopidogrel treatment at rest suggesting reduced platelet activation in vivo [215], but this may have been confounded by a high pretreatment value in the clopidogrel group.

Clopidogrel treatment reduced the PLA response to in vitro stimulation by 1 μ M ADP by $62\pm 5\%$ before and $40\pm 4\%$ after exercise ($p<0.001$ for both). ADP enhanced the formation of platelet-conjugated neutrophils, monocytes and lymphocytes in vitro, and these responses were antagonized by clopidogrel ($p<0.001$). The ANOVA showed an overall effect of clopidogrel treatment on thrombin responsiveness ($p<0.05$) but no attenuation of the exercise effect ($p=0.25$) by clopidogrel compared to placebo.

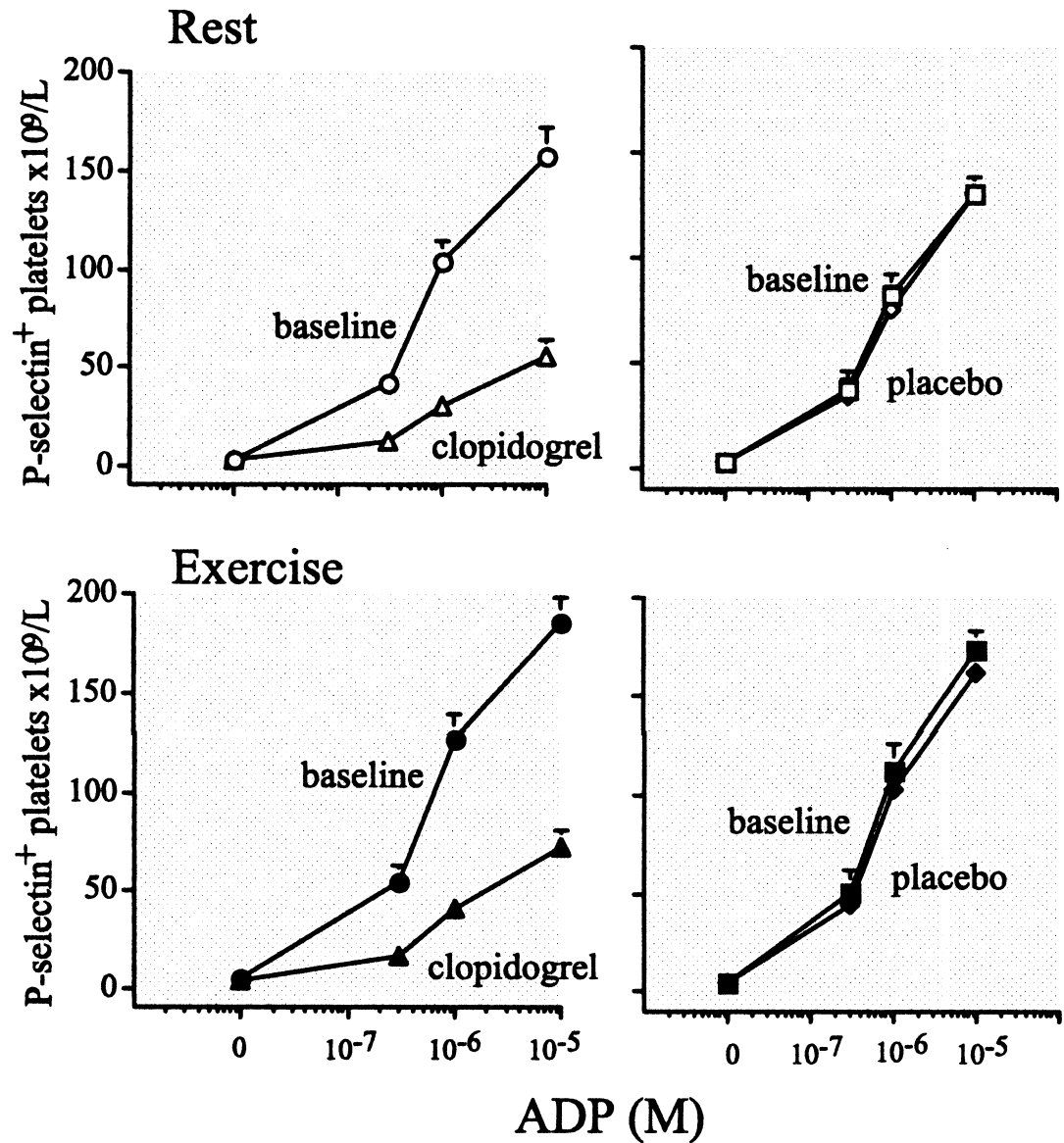


Figure 9: Platelet activation (P-selectin positive platelets) upon stimulation in vitro by ADP. Without treatment (baseline) and during co-treatment with clopidogrel (left hand panels) or placebo (right hand panels). The upper panels are from before exercise (rest) and the lower panels are after exercise. As expected, clopidogrel treatment attenuated the responses to ADP ($p < 0.001$), but did not significantly attenuate the enhancing effect of exercise ($p = 0.43$ by ANOVA).

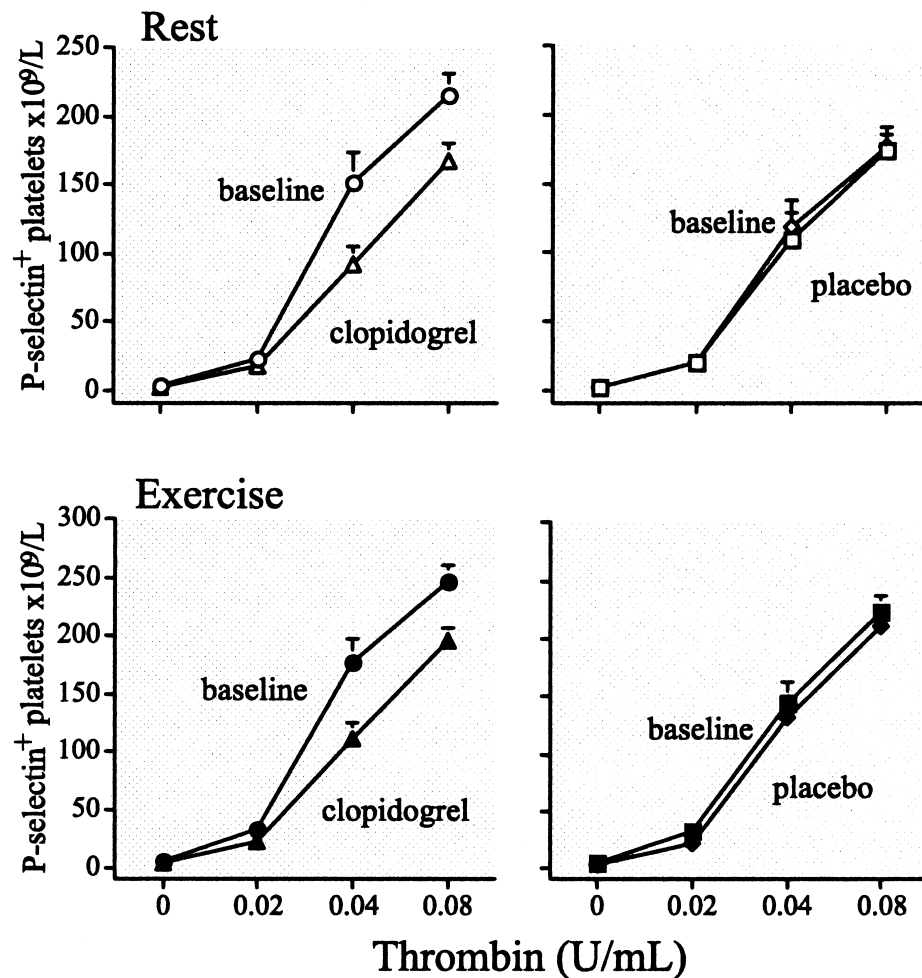


Figure 10: Platelet activation (P-selectin positive platelets) upon stimulation in vitro by thrombin. Without treatment (baseline) and during co-treatment with clopidogrel (left hand panels) or placebo (right hand panels). The upper panels are from before exercise (rest) and the lower panels are after exercise. Clopidogrel treatment attenuated the responses to thrombin ($P < 0.001$), but did not significantly attenuate the enhancing effect of exercise ($p = 0.31$ by ANOVA). The inhibitory effect of clopidogrel on responses to thrombin presumably reflects inhibition of the positive feed-back caused by ADP released from activated platelets.

The ECG evaluation of ambulatory or exercise-induced ischemia showed no effect of clopidogrel treatment. Neither of the inflammatory markers CRP, IL-6 or sCD40L, were affected by clopidogrel compared to placebo treatment.

In summary, clopidogrel treatment inhibited platelet and PLA responses to ADP and thrombin stimulation (i.e. platelet released ADP) in vitro but the acute prothrombotic response to exercise was little influenced by clopidogrel treatment in aspirin treated CAD patients. This indicates that the P2Y₁₂ receptor is not likely to be of importance in stress-induced platelet activation caused by heavy exertion. Furthermore, we found no support for the hypothesis that release of vasoactive substances from activated platelets contributes to myocardial ischemia in patients with stable angina pectoris and CAD documented by coronary angiography.

4.6 GENERAL DISCUSSION

Antiplatelet therapy with aspirin and clopidogrel is a cornerstone in cardiovascular prevention and lowers cardiovascular mortality and morbidity in patient with CAD [16,17]. Mental stress and physical exertion can trigger acute coronary events [56] and prothrombotic responses to stress may contribute to such triggering [11-13,56]. Hemodynamic factors that increase wall stress and inflammatory weakening of the fibrous cap of the plaque may result in a plaque rupture exposing a highly thrombogenic surface [5]. Activated platelets and coagulation seem to contribute to accelerated atherosclerosis [14,216].

The work presented in this thesis shows that both aspirin and clopidogrel has limitations as antithrombotic agents. Recurrent ischemic events occurrence during dual antiplatelet therapy including stent thrombosis remains a major clinical problem [21]. Numerous pathways are involved in platelet activation and platelets contain storage granules with large amounts of prothrombotic, angiogenic, proinflammatory and proatherogenic substances released upon activation [87].

In a previous study a single high dose of aspirin failed to attenuate the prothrombotic responses to exercise [195] and in the present work neither clopidogrel alone nor the addition of clopidogrel to aspirin treatment influenced the prothrombotic responses to exercise. Adrenaline can overcome the effects of aspirin in animal models of arterial thrombosis [217] and noradrenaline infusion increases platelet aggregability despite aspirin in humans [92]. β_1 -blockade attenuates the haemodynamic responses to exercise but there was no effect on the platelet activation response in patients with stable angina [9]. Thus, sympatho-adrenal activation may be of importance, but the exact mechanisms responsible for stress-induced platelet activation remain illusive.

Since clopidogrel treatment attenuates platelet activity in vivo at rest but exercise counteracts the platelet stabilizing effects of clopidogrel in both healthy volunteers and in patients with CAD the hypothesis that ADP and the platelet P2Y₁₂ -receptor is involved in stress-induced platelet activation was not supported. However the relative inhibition of ADP induced platelet activation by clopidogrel was similar at rest and following exercise indicating that treatment protects against thrombotic events also during stress.

There is currently considerable interest in issues related to clopidogrel resistance and variability of responses to clopidogrel treatment [21,218]. In the present study of CAD patients the mean degree of inhibition of ADP-induced platelet activation was 64% but the responses varied considerably between patients (from 22-87% inhibition) in agreement with previous findings [185,218,219]. In healthy volunteers there was less variation as the degree of inhibition of ADP-induced activation was 72 % (range 54-85%). The variable degree of inhibition of ADP-induced platelet responses by clopidogrel treatment may have influenced the possibilities of detecting attenuation of platelet activation by heavy exercise, especially in the CAD patients. Therapeutic failure manifested by ischemic events has been associated with high on-treatment

platelet reactivity [20]. The absolute level of platelet reactivity during treatment is a better predictor of thrombotic risk than responsiveness to clopidogrel [21].

A wide range of “aspirin resistance” or poor responsiveness has been reported [22]. In a direct comparison of different functional tests Lordkipanidzé et al found a range that “aspirin resistance” varied between 2.8-59.5 % [115]. Several authors have reported an increased risk of suffering cardiovascular events [22,137,148,149,220,221] among such patients. One explanation for this result may be our finding that there is a recovery of platelet function 24 hours after dosing and that pronounced AA-induced platelet aggregation could still occur in whole blood despite aspirin treatment. Of interest, Spectre et al found that twice daily dosing of aspirin improves platelet inhibition in whole blood (AA-induced aggregation) in patients with type 2 diabetes [222].

There is residual AA-induced platelet activation in patients treated with aspirin. Frelinger et al found that this activation was independent of COX-1 and COX-2 and mediated in part by ADP induced platelet activation. This finding raises the possibility that clopidogrel treatment in addition to its direct antiplatelet effect via the P2Y₁₂ ADP receptor also decreases residual arachidonic acid induced platelet activation and that this may have clinical implications [223]. Platelet activation by ADP should increase AA-release acting as a positive feed-back for platelet activation in a similar manner as the ADP-mediated positive feed-back discussed above. Indeed, several authors have shown that clopidogrel treatment reduces thromboxane production in vivo and ex vivo [224,225]. In our study in healthy volunteers (paper IV), however, clopidogrel did not influence either plasma or urinary 11-dehydro-TxB₂. This may be due to already low thromboxane generation in healthy subjects in contrast to patients with manifest vascular disease where thromboxane generation is increased [4,55]. However, Helgasson et al had similar negative findings as ours in a study in 30 chronic ischemic stroke patients treated with aspirin and clopidogrel [226]. It has recently been proposed that aspirin may provide little additional protection against athero-thrombotic events in presence of strong P2Y₁₂ receptor blockade [227], but this is speculative since no large study has investigated the effect of adding aspirin or placebo to clopidogrel treatment in patients with CAD. Of note, abundant data indicate that stopping aspirin in patients with strong need for double antiplatelet drug treatment, such as patients with drug eluting coronary artery stents, increases the risk of stent thrombosis [228].

The present work has improved the methodology for immunological measurements of 11-dehydro-TxB₂ in urine, and has also shown that such measurements may be performed in plasma for the evaluation of platelet activity. Interestingly, conventional analysis of urinary 11-dehydro-TxB₂ excretion has been shown to predict future complications in aspirin treated patients at high risk of cardiovascular disease [221]. Whether our optimized method may be even more efficient with respect to prognostic information in patients with risk of cardiovascular complications is not known.

There is no “gold standard” with regard to platelet function testing and a combination of several methods probably provides the most reliable results. Using urinary 11-dehydro-TxB₂ and whole blood aggregometry may be a good approach for the evaluation of in vivo effects of aspirin treatment. Measurements of AA-induced platelet aggregation in PRP and serum TxB₂ may provide the most specific assessment of

platelet COX inhibition, but more “global” markers of platelet activity may reflect the clinical consequences of antiplatelet therapy better [21,229]. Work to improve and evaluate global bedside tests should be intensified as clinical research should aim at refining treatment and improving prognosis in patients; global tests seem to be the right path to choose in this respect.

The relationship between surrogate markers for platelet function and clinical events is a critical issue determining the dosing regime. The time dependence of inhibitory effects should be considered and the interval between dosing and sampling has to be taken into consideration. The ability of aspirin to block platelet COX-1 (“true aspirin resistance”) should be clearly distinguished from treatment failure due to the importance of other disease mechanisms or factors such as poor compliance, non-specific measurements, too long an interval since the last dose, and/or too low a dose when discussing “aspirin resistance” [19,147].

Aspirin and clopidogrel form cornerstones in the treatment of cardiovascular disease. The absolute benefit and proportional effects of antiplatelet prophylaxis with low dose aspirin are comparable with those of blood pressure lowering with antihypertensive drugs and lipid-lowering with statins [137]. Cardiovascular disease is multifactorial and it is not surprising that only about 25 % of all vascular complications can be prevented by aspirin [55]. However, defining the optimal aspirin dosing interval for each patients group and establishing well documented means of monitoring individual patients may further improve the prevention of cardiovascular disease by antiplatelet drugs. The present thesis shows some limitations of aspirin and clopidogrel and also suggests a treatment regimen that deserves to be tested in clinical studies of cardiovascular disease, i.e. low dose aspirin twice daily.

5 SUMMARY AND CONCLUSIONS

The following conclusions may be drawn from the studies:

- Measurements of 11-dehydro-TxB₂ in urine and plasma are of considerable interest when evaluating platelet activity in vivo. An improved sample work-up procedure combined with a modified commercially available EIA has been developed and evaluated. A key feature of the assay is to keep 11-dehydro-TxB₂ in its open ring form during the entire assay by equilibrating at pH 8.6 which improved the recovery in and sensitivity of the assay. Large clinical studies using this optimized methodological approach are, however, lacking.
- Aspirin provides weak inhibition of platelet aggregation in whole blood with normal calcium levels, despite effective platelet COX inhibition. There is recovery of thromboxane-dependent platelet activation in whole blood within a normal 24 h dosing interval during aspirin treatment. Considering that the incidence of myocardial infarction is highest in the early morning hours, i.e. when antiplatelet effects of last aspirin dose usually are the lowest, this finding may be of clinical importance.
- Several factors have to be taken into consideration when assessing platelet function; the anticoagulant used may artifactually modify platelet responses, and it is important to distinguish between measurements of specific treatment effects (i.e., whether the “drug target” is effectively inhibited) and more non-specific or “global” measurements of platelet activation which may be of pathophysiological and prognostic importance. Measurements in whole blood are more physiological than measurements in PRP, and may provide clinically relevant information.
- There is no single “gold standard” platelet function test for the monitoring of aspirin treatment. Urinary 11-dehydro TxB₂ and whole blood aggregation may provide valuable estimates of “global” in vivo effects of treatment, whereas AA-induced aggregation in PRP and S-TxB₂ provide specific data on platelet COX-inhibition.
- Compared to healthy pregnant women, patients at high risk of suffering preeclampsia have an increased thromboxane formation, as assessed by 11-dehydro-TxB₂ excretion, already before gestational week 13. Low-dose aspirin treatment (75 mg once daily) effectively inhibits platelet-dependent thromboxane production early in pregnancy.
- Clopidogrel treatment attenuates platelet activity in vivo at rest and during exercise, but ADP is not important for the enhancement of platelet reactivity by exercise in healthy volunteers or in aspirin treated patients with CAD.
- Addition of clopidogrel to aspirin treatment did not attenuate either exercise induced or ambulatory ischemia in coronary artery disease patients.

- Individualized antiplatelet therapy, based on point-of-care platelet function tests that are well validated, may improve cardiovascular prevention by allowing the tailoring of doses and dose intervals of the antiplatelet agents used to the needs of the individual patient. However, this approach must also be shown to be beneficial regarding clinical outcomes before general use.

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